DISSERTATION

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The effect of alkaloid harmine, emetine, and sanguinarine on human cancer cells

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ZUSAMMENFASSUNG

Über 21000 Alkaloide konnten bisher identifiziert werden. Hiermit stellen sie die größte Gruppe der stickstoffhaltigen Sekundärstoffe dar. Viele dieser Alkaloide sind für Tiere oder Menschen giftig. Die medizinische Nutzung von Alkaloiden kann als Ausnutzung von Eigenschaften angesehen werden, die eigentlich aus ökologischen oder evolutionsbedingten Gründen entwickelt wurden. Während der letzten Jahrzehnte rückte vor allem das krebsbekämpfende Potential der Alkaloide in das Zentrum des Interesses. Mehrere Alkaloide werden seit über 40 Jahren als Krebsmedikamente genutzt.

In dieser Studie wurde die Cytotoxozität der drei Alkaloide Harmin, Emetin und Sanguinarin in den menschlichen Krebszelllinien MCF-7, HeLa und SiHa ermittelt. Die Ergebnisse des MTT-Assays zeigten, dass diese Alkaloide eine zelluläre Cytotoxizität aufweisen und einen zeit- und dosisabhängigen Zellzyklusarrest induzieren. Telomere und Telomerasen stellen aufgrund ihrer speziellen Struktur oder ihrer krebsrelevanten Eigenschaften interessante Ziele für die Krebsforschung dar. Es konnte nachgewiesen werden, dass einige natürlich vorkommende Alkaloide die Aktivität der Telomerase inhibieren. In unserer Studie wurde zuerst untersucht, ob Harmin, Emetin und Sanguinarin menschliche Telomerase inhibieren können. Durch den TRAP-Assay konnte nachgewiesen werden, dass alle drei Stoffe die Telomeraseaktivität in den Zelllinien inhibieren können, wenn die Zellüberlebensrate nach der Behandlung auf 70% reduziert wurde. Ein Vergleich der Inhibitionsrate aller Alkaloide zeigte, dass Harmin eine stärkere Inhibition aufwied als Emetin oder Sanguinarin. Um die zugrunde liegenden Mechanismen zu verstehen wurde Harmin einer näheren Untersuchung in den zwei Krebszelllinien MCF-7 und HeLa unterzogen. Hierdurch konnten wir nachweisen, dass Harmin zwar in beiden Zelllinien die Telomeraseaktivität signifikant herabsetzt, aber die zugrunde liegenden Mechanismen durchaus unterschiedlich sind. Harmin induzierte eine Herunterregulierung der Expression von hTERT mRNA in MCF-7-Zellen. In HeLa-Zellen verursachte Harmin das alternative Splicing von hTERT, begleitet von einem Anstieg der nichtfunktionellen β-Splice-Form. Es wurde bereits gezeigt, dass Harmin DNA-Schädigungen hervorruft. Diese Resultate konnten wir in unserer Studie bestätigen. Durch ein β-Galactosidase-Staining und mehrere Western-Blot-Analysen konnten wir beobachten, dass die kontinuierliche Gabe von Harmin DNA-Schädigung auslöst. Die behandelten MCF-

7-Zellen alterten schneller durch den p53/p21-Pathway. Zusammenfassend legen unsere Daten nahe, dass die Cytotoxizität von Harmin zumindest teilweise durch die Inhibition der menschlichen Telomerase bedingt ist.

SUMMARY

Over 21,000 alkaloids have been identified, which thus constitute the largest group among the nitrogen-containing secondary metabolites. Many alkaloids have shown their powerful toxicity towards animals or humans. The medicinal use of alkaloids could be regard as an exploitation of properties that originally had been selected and developed in an ecological or evolutionary context. During the past decades, more attention has been drawn on their anticancer potencies. A number of alkaloids have been used as anticancer drug over 40 years.

In this study, the cytotoxicity of three alkaloids harmine, emetine, and sanguinarine were selected and evaluated in human cancer cells including breast cancer cell MCF-7, cervical cancer cell HeLa and SiHa. Results obtained from MTT assay showed that these alkaloids exhibited cellular cytotoxicity against human cancer cells and induce cell cycle arrest in dose- and time- dependent manner. Telomeres and telomerase have become interesting targets for anticancer research based on their special structure or cancer-associated character, some natural alkaloids have been identified are able to inhibit telomerase activity. In our study, we firstly investigated whether the alkaloid harmine, emetine, and sanguinarine were able to inhibit human telomerase. Data obtained from TRAP assay indicated that when the cell viability of each cell line was remaining around 70% after the treatment of each drug, all the compounds exhibited an inhibitory effect on human telomerase. Compared the inhibitory rate between each alkaloid, harmine initiated a greater reduction than that of emetine or sanguinarine. To elucidate the underlying mechanisms, harmine was especially selected and applied in parallel in two cancer cell lines MCF-7 and HeLa in further research. We have found that although harmine could significantly inhibit the telomerase activity in both cell lines, the mechanisms were quite different. Harmine induced a down-regulation of the expression of hTERT mRNA in MCF-7 cells, whereas it regulated the hTERT alternative splicing accompanied by an increase of the non-functional β splice form in HeLa cell. Harmine has been documented is able to trigger DNA damage, we have obtained the consistent results in our study as well. By applying β-galactosidase staining and a series of western blotting analysis, we observed that the chronic treatment of harmine initiated a DNA damage response, and the treated MCF-7 cells eventually entered an accelerated senescence status through p53/p21 pathway. Taken together, our data suggest that the cytotoxicity of harmine might be generated, at least partially, by the inhibitory effect on human telomerase.

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LIST OF ABBREVIATIONS

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%	Percent
ºC	Celsius degree
µg, µl, µM	Micro-gramm, -liter, -molar
A	Absorbance
aa	Aminoacid
ALT	Alternative lengthening of telomeres
APS	Ammonium persulfate
BrdU	Bromo deoxyridine
BSA	Bovine serum albumin
β-actin	beta actin
cDNA	copie-deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethyl -ammonio]-1-propanesulfonate
CO2	Carbon dioxide
CP	crossing point
Da	dalton
dNTP	Deoxynucleotide 5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
DNA	Dexyribonucleic acid
dsDNA	double strand DNA
FBS	Fetal Bovine serum
h	Hour
HeLa	Henrietta Lacks, cervical carcinoma cell line
hTERT	Human Telomerase catalytic subunit
hTR	Human telomerase RNA
MCF-7	Human breast adenocarcinoma cell line
min	Minute
ml	Milli-liter
mM	Milli-molar
mRNA	Messenger ribonucleic acid
nm	nanometer
MTT	3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Hydrogen-ion-concentration
PI	Propidium Iodide
PMSF	Phenylmethanesulphonylfluorid
RNA	Ribonucleic acid
RNase	Ribonuclease
RNasin	Ribonuclease inhibitor
rpm	Rotation per minute
RT	Reverse transcription
RT-PCR	Reverse transcription-PCR
qPCR	quantification-PCR (Real-Time PCR)
SDS	Sodiumdodecylsulphate
SD	Standard deviation
Sec	Second
Taq	Termophilus aquarius
TEMED	N,N,N,N,-Tetra-methyl-ethylen-diamine
TRAP	Telomerase Rpeat Amplification Protocol
Tris	Tris-(hydroxymethyl)-aminonethan
Triton X-100	Octylphenol-polythylenglycol ether
Tween-20	Polyoxyethylen-sorbitan-monolaurat
u	Unit
UV	Ultraviolet light
V	Volt
WB	Western blot
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactoside

1 INTRODUCTION

1.1 ALKALOIDS

1.1.1 Introduction

Plants produce plenty of secondary metabolites (SM), the alkaloids are one of the most diverse groups of SM found in living organisms and have a series of structure type (Roberts and Wink, 1998; Wink, 2007; 2008). The first alkaloid, morphine, was identified from the opium poppy, *Papaver somniferum* by Sertürner in 1806 (Kutchan, 1995). To date, over 21,000 alkaloids have been identified (Wink, 2007).

1.1.2 Classification of alkaloids

Alkaloids are typical nitrogen containing ring structures. The position of nitrogen can be different depending on the class of alkaloids. In general, they have definite melting points and have a bitter taste. Compared with other natural products, alkaloids can be easily isolated due to their structure in which the nitrogen makes the compound basic, and the compounds exist in plants as a salt. Thus, alkaloids are often extracted with water or mild acid and then recovered as crystalline material after treatment with a base (Manske and Holmes, 1965).

Alkaloids are a diverse group of chemical constituents. The criteria currently for alkaloid classification are: the structure and other chemical features of the alkaloid molecule, the biological origin, and the biogenetic origins (Roberts and Wink, 1998). There is no unified, taxonomy principle to classify all alkaloids, but it is possible to recognize four groups (Roberts and Wink, 1998):

1. Alkaloids derived from amino acids such as arginine, lysine, tryptophan, and so on.
2. Purine alkaloids, such as the xanthine caffeine
3. Aminated terpenes, e.g., the diterpene aconitine or the triterpene solanine
4. Polyketide alkaloids where nitrogen is introduced into a polyketide carbon skeleton as in coniine and the coccinellines

Alkaloids constitute the largest group among the nitrogen-containing SM including 700 non-protein amino acid, 100 amines, 60 cyanogenic glycosides, 100 glucosinolates, and 150 alkylamides (Wink, 2007; 2008). Alkaloids are widely distributed in plant kingdom, especially within the flowering plants. Recently, an increasing number of them have been found in animals, insects, marine organisms, microorganisms, and lower plants (Wink, 1993;

1999a; 1999b). The distribution of alkaloids are restricted in plants, microbial, animal species, and the organisms where these constituents have their own genetically defined alkaloid pattern, which may be useful under certain circumstances as chemical characters in systematic (Wink, 1988; 2008; Roberts and Wink, 1998).

1.1.3 Cytotoxicity of alkaloids and the associated molecular modes of action

1.1.3.1 Cytotoxicity of alkaloids

Many alkaloids have shown their powerful toxicity on animals or humans. Most of the lethal alkaloids fall into the class of neurotoxins (Wink, 2008). And some act on the other different organs. For example, pyrrolizidine alkaloids which occur in *Senecio* plants will cause hepatic disease within a few weeks in horses and cattle (Wink, 1993; Elliott *et al.*, 2005). The steroidal alkaloids induce embryo mortality in two fish species due to structural or functional abnormalities in the early development stages (Crawford and Kocan, 1993). A summary of cytotoxic properties of alkaloids was reviewed (Wink, 1993; 2007). Vinblastine, vincristine, colchicine and taxol are particularly famous in their toxicity on the mitotic spindles (Wink, 2007), and they have been used in chemotherapy. A cytotoxic effect can also be generated when cell membranes become leaky (Wink, 2008). In general, biomembranes are almost impermeable for ions and polar molecules, such prevention can be achieved through specific transport proteins including ion channels, pores, or carrier proteins (Roberts and Wink, 1998). If the transport of metabolites has to proceed against a concentration gradient, the transport processes must be directly or indirectly energized. These complex transport systems have become targets of many natural products (Roberts and Wink, 1998).

1.1.3.2 Molecular modes of action

Alkaloid cytotoxicity can be generated through interference with important molecular targets present in a cell (Fig.1). The modulated molecular target can negatively influence its communication and the associated network. Subsequently, the metabolism and the function of cells, tissues, organs, and even the complete organism can be literally affected and an overall toxic effect occurs (Wink, 2008). The main targets include DNA, RNA and some associated enzymes, protein biosynthesis, protein conformation, biomembranes and membrane proteins (Wink, 2007).

1.1.3.2.1 Specific interactions

Compared with plants, the nerve cells are unique cells present in animals. Therefore, neuronal signal transduction, and the endocrinal hormone system can be targeted by plants particularly (Wink, 1993;2000;2008). Compounds that interfere with these targets are usually not toxic for the plants producing them. Many alkaloids were characterized as specific modulators and have been modified during evolution. The modification enable these compounds mimic endogenous ligands, hormones, or substrates (Wink, 1988;1993;2000). Some deadly alkaloids are strong neurotoxins that were selected for defense against animals (Wink, 1993;1998;2000; Roberts and Wink, 1998). A summary of the potential neuronal targets that can be affected by alkaloids is available in the review (Wink, 2008).

Neurotransmitters derive from amino acids, and most of them are amines which become protonated under physiological conditions (Wink, 2008). It has been demonstrated that several alkaloids have structural similarities to those neurotransmitters due to alkaloids also derive from amino acids. Thus, they can be considered as neurotransmitter analogs (Wink, 2008). These structural neurotransmitter-like alkaloids can bind to neuroreceptors to activate or inactivate them. In addition, some ion channels, such as the Na^+, K^+, and Ca^+ are also important targets, they have been reported to be inhibited or induced by several known alkaloids (Wink, 1993;2000;2008). Neuronal signal transduction is very critical in an animal, any disturbance can lead to organ's dysfunction, such as mind-altering, or hallucinogenic properties (Wink, 1993; Roberts and Wink, 1998).

1.1.3.2.2 Interactions with DNA, RNA, and associated enzymes

1) DNA alkylation and intercalation

As DNA encodes all RNAs, proteins which are important for metabolism and development of an organism, therefore, DNA is an highly attractive target (Wink, 1993; Roberts and Wink, 1998). The reaction can happen in two patterns: DNA alkylation and intercalation. Alkylating agents can form covalent bonds with the host DNA onto N_6 guanine, which eventually lead to DNA strand breaks (DSBs). These molecules include cisplatin, nitrosourea derivatives and nitrogen mustard. DSB can be repaired by the enzyme alkylguanine-DNA alkyl transferase (AGT) through a transferring action in which the alkyl group could be removed from the lesions stoichiometrically to a cysteine in its active site (Vaidyanathan and Zalutsky, 2004).

If the repair process fails, mutation may happen, consequently, transitions or transversions may occur within nucleotides.

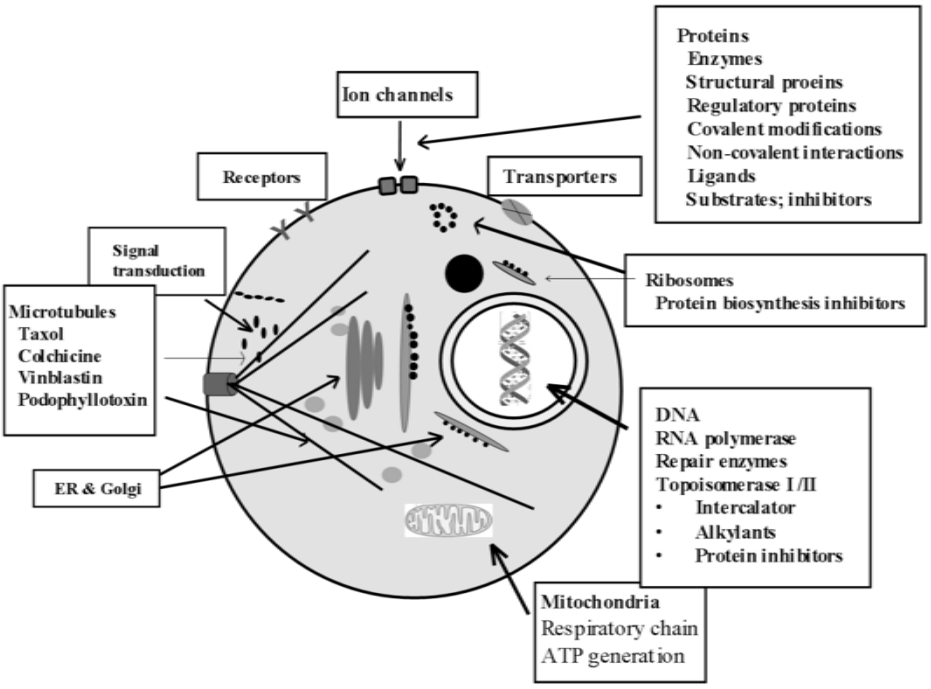


Fig 1. Molecular targets in animal and human cells that can be affected by natural products (Wink, 2007).

Intercalation is a non-covalent reaction between small molecules and DNA. Small molecules that intercalate between stacks of DNA bases have been investigated for more than 40 years, due to the activity of such molecules as therapeutic drugs or as mutagenic agents (Persil and Hud, 2007). Many of intercalating compounds are planar and lipophilic, which facilitate these compounds insert between stacks of base pairs and stabilize the double stranded DNA, whereby to disturb DNA replication or transcription (Wink, 2007). The intercalation also takes place in RNA. RNA is basically single stranded. However, most RNA molecules have double-stranded stem structures due to complementary base pairing. These double stranded regions can also be intercalated (Wink, 2007). Intercalation often induces a frameshift mutation, which changes the sequence of corresponding amino acid to trigger some detrimental effect on the individual cell or organ (Wink, 2007). Some toxic alkaloids have intercalating characters, such as isoquinoline, quinoline, and indole alkaloids, which are synthesized from the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Wink *et*

al., 1998; Wink, 1999b). A review of alkaloids that interfere with DNA, RNA, and associated enzymes is available (Wink, 2007).

2) DNA topoisomerase I and II

DNA Topoisomerase is an enzyme that alters the supercoiling of double-stranded DNA. DNA Topoisomerase acts by transiently cutting one (topoisomerase I) or both strands (topoisomerase II) of the DNA. DNA topoisomerase I and II are ubiquitous enzymes that play an essential role in transcription, replication, chromosome segregation, and DNA repair. The inhibitions of these enzymes usually result in cell-cycle arrest and cell death by apoptosis (Wink, 2007). Topoisomerase I is an important target in tumor cell, it can be targeted by some alkaloids, such as quinazoline–quinoline (Ma et al. 2004), coralyne (Gatto *et al.*, 1996), and pallidine (Cheng *et al.*, 2008) which turn these enzymes into cellular poisons.

The mechanism of the inhibition of topoisomerase caused by compounds has been documented. These compounds either directly inhibit its catalytic activity (Ganguly *et al.*, 2007; Chene *et al.*, 2009) or stabilize the transient “cleavable ternary complexes” by preventing strand resealing (Wink, 2007), whereby the cleavable complexes are converted to lethal lesions when the cell tries to use the damaged DNA tempelate for replication (Denny, 1997; Bailly, 2000). Data obtained from different groups showed that the DNA intercalating alkaloids cryptolepine (Bonjean *et al.*, 1998; Dassonneville *et al.*, 1999), matadine, and serpentine can tightly bind DNA. These three compounds stabilize the DNA-topoisomerase II complex and stimulate the cutting of DNA by topoisomerase II (Dassonneville *et al.*, 1999; Wink, 2007). This observation supports the 'drug stacking' model of interaction, where inhibitors with a 'deep intercalation mode' are responsible for topoisomerase I-mediated cleavage and those with an 'outside binding mode' are responsible for topoisomerase II-mediated cleavage (Denny, 1997). A number of alkaloids affect both enzymes, such as benzophenanthridine (Okamoto *et al.*, 2005), indenoquinolone, and acridine alkaloids (Denny, 1997; Wink, 2007).

3) Telomeres and telomerase

The ends of linear eukaryotic chromosomes contain specialized structures termed telomeres. Human telomeres consist of tandem repeats of the hexameric sequence TTAGGG. These repeats help maintain chromosomal integrity and prevent them from degradation, recombination, and being mistaken for DNA double-strand breaks (Shay and Wright, 2005). A loss of telomeric DNA is found with each cell division due to the end replicative problem (Watson, 1972) and eventually lead to replicative senescence. Almost all eukaryotic cells depend on telomerase, a ribonucleoprotein enzyme responsible for adding telomeric DNA onto 3' ends of chromosomes (Greider and Blackburn, 1985) for the *de novo* synthesis of telomeres.

Telomeres and telomerase become interesting targets for anticancer research based on their special structure or cancer-associated character. Recently, some natural alkaloids were found to able to inhibit telomerase. These alkaloids include ellipticine (Garbett and Graves, 2004), cryptolepine, neocryptolepine (Guittat *et al.*, 2003; Wink, 2007). These alkaloids affect targets either by triggering a DNA damage response via "drug stacking" model of interaction with telomeres, or by inhibiting of telomerase enzymatic activity. More details have been described in chapter 1.2.

1.1.3.2.3 Cytoskeleton

The cytoskeleton of eukaryotic cells pervades the cytoplasm. It consists of three broad classes of proteins: actin filaments, microtubules and intermediate filaments. The regulation of cytoskeletal activity plays a role in cell maintenance, cell division, and apoptosis. During cell division, the duplicated chromatids are separated and pulled apart into daughter cells; such process is completed by a complex interaction of microtubules. Microtubules are elements of cellular cytoskeleton and are polymers of tubulin (Wink, 2007). It has been described that GTP promotes protofilaments forming; several protofilaments are assembled into microtubules. On the other hand, the microtubules are disassembled when GDP is dominant (Wink, 2007). Any alteration on microtubule dynamics leads to cell cycle arrest at mitosis and eventually apoptosis (Zhou and Giannakakou, 2005). The cell division in cancer cells is faster than differentiated normal cells. Therefore, to inhibit the activity of microtubule becomes a major target for anticancer agents (Wink, 2007). These drugs have a wide spectrum of activity against hematological and solid tumors. It has been demonstrated that

most of anticancer drugs that come from nature can interfere with microtubules (Wink, 2007). The bis-monoterpene indole alkaloids vinblastine and vincristine are well-known examples. The interactions of these alkaloids with tubulin heterodimers and microtubules have been studied extensively; they can interfere with microtubules dynamics in terms of special binding sites (Lobert *et al.*, 1996; Wink, 2007).

1.1.3.2.4 Induction of apoptosis

A number of toxins have been identified are able to induce apoptosis. The apoptosis appears to be the major mechanism of cytotoxicity caused by SM. These SM include polyphenols, terpenoids, and also alkaloids (Wink, 2007). Those SM with apoptotic properties have drawn much attention in anticancer drug development. As documented, several protoberberine and benzophenanthridine alkaloids, indole alkaloids induce apoptosis (Wink, 2007). These compounds usually intercalate DNA, and subsequently inhibit DNA and RNA polymerase, topoisomerase, and even ribosomal protein biosynthesis (Wink *et al.*, 1998; Wink, 2007), or bind to tubulin/microtubules to behave as spindle poisons.

1.1.3.2.5 Interactions with ABC transporters and cytochrome p450

In order to overcome the intestinal and blood brain barrier, plants have evolved lipophilic toxins that can enter body by diffusion. On the opposite side, animals have developed powerful detoxification mechanisms to cope with these toxins (Wink, 2007). One of the mechanisms is mediated by an enzyme family, known as cytochrome p450 oxidase. This pathway consists of two phases. In phase I, a group of enzymes, known as cytochrome p450 oxidases (CYP) act on lipophilic toxins by introducing hydroxyl groups to make them more hydrophilic (Guengerich, 2001). In phase II, the hydroxylated compounds are conjugated with polar molecules. Then these conjugates are eliminated by the kidneys and urine.

Another well-known pathway is mediated by the ATP binding cassette (ABC) transporters. These transporters are present in most of cells and especially active in intestinal, liver, kidney, and endothelial (Nielsen and Skovsgaard, 1992; Nooter and Stoter, 1996). These ATP-driven transporters can pump lipophilic compounds out of cell, either back to the gut lumen or into the blood system. In such a way, the intracellular concentration of potentially toxic compounds can be reduced (Wink, 2007).

1.1.4 The chemotherapeutic effect of alkaloids

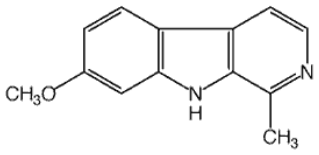
Cancer comprises a large variety of malignant tumors that can affect nearly all organs of the body. From a primary tumor, metastases can spread to other tissues and organs, which is fatal if a tumor destroys the function of a vital organ. Treatment includes surgery, radiotherapy and chemotherapy. In the clinic, doctors and researchers conduct clinical research in distinct segments called phases. Each phase of a clinical trial is designed to provide different information about the new treatment, such as the dose, safety, and efficacy. After promising treatments are explored in animal and/or laboratory studies, researchers perform clinical trials. There are three phases for clinical trials. The goal of a phase I clinical trial is to prove that a new drug or treatment, which has proven to be safe for use in animals, may also be given safely to people. Phase II clinical trials are designed to provide more detailed information about the safety of the treatment, in addition to evaluating its efficacy. The goal of phase III clinical trials is to take a new treatment that has shown promising results among a small number of patients with a particular disease and compare it with the current standard for that specific disease. In this phase, data are gathered from large numbers of patients to determine whether the new treatment is more effective and possibly less toxic than the current standard treatment.

Cancer cells usually divide much faster than ‘normal’ cells. Therefore, compounds that stop cell division (e.g. alkaloids, such as vinblastine, vincristine, paclitaxel, docetaxel, camptothecin, colchicine, demecolcine, or the lignan podophyllotoxin) or cytotoxic compounds that directly kill cancer cells are the mainly employed means of chemotherapy (Roberts and Wink, 1998; Wink *et al.*, 2005; Wink, 2007). Whereas vinblastine, demecolcine and podophyllotoxin inhibit the polymerization of tubulin to microtubules, paclitaxel stabilizes the microtubule complexes. Camptothecin mainly acts as an inhibitor of DNA topoisomerase I, the podophyllotoxin derivatives (such as etopophos) inhibit topoisomerase II. Inhibition of polymerization or dissociation of microtubules and DNA topoisomerase stop the multiplication of healthy and tumour cells (Wink *et al.*, 2005; Wink, 2007). To date, more than 50 drugs are available for cancer therapy, nevertheless, the need for more effective anticancer agent remains.

1.1.5. Alkaloids in medicine

1) Harmine

Harmine is the most representative naturally occurring β-carboline alkaloid, which was originally isolated from *Peganum. harmala L*, mainly occurring in seeds and roots (Budavari, 1989; Roberts and Wink, 1998; Wink and Wyk, 2008). More recent study showed that harmine is found as 4.3 % (w/w) in dry seeds and as 2.0 % (w/w) in root, respectively (Herraiz *et al.*, 2009).



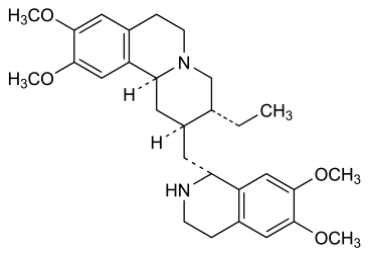
harmine

Harmine is inhibitor of monoamine oxidase (MAO) (Kim *et al.*, 1997; Roberts and Wink, 1998), which has been identified as early as 1965 (Burger, 1965). MAO is an enzyme essential for the metabolism of neurotransmitters. Harmine is a known hallucinogenic substance since it binds to serotonin receptor, and whereby to enhance serotonin activity (Roberts and Wink, 1998; Wink, 2007; Wink and Wyk, 2008). Plants containing it are famous drugs in S America (including ayahuasca) (Wink and Wyk, 2008). In vitro studies showed that harmine can intercalate DNA (Taira *et al.*, 1997; Roberts and Wink, 1998; Wink, 2007), and in such a way to compromise DNA replication, or to inhibit the associated enzymes such as topoisomerases (Funayama *et al.*, 1996; Wink, 2007). More specific research demonstrated that harmine inhibits the activity of topoisomerase I, whereas it has no effect on topoisomerase II, even at a high concentration of 600 μM (Cao *et al.*, 2005; Wink, 2007; Jimenez *et al.*, 2008). It has been reported that harmine inhibited several cyclin-dependent kinases such as Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25 (Song *et al.*, 2002; Song *et al.* 2004), suppressed the growth of *Leishmania* parasites with a moderate selectivity (Di-Giorgio *et al.* 2004), and acts as a cell-type-specific regulator of the key adipogenic factor PPARγ (Waki *et al.*, 2007).

2) Emetine

Emetine is the main alkaloid in C*ephaelis acuminata*, which belongs to isoquinoline alkaloids group. Emetine has been used as a second choice in the treatment of severe intestinal amoebiasis and hepatic amoebiasis since 1912 (Vedder, 1912). It is also used in low dose as an expectorant drug with secretolytic and secretomotoric properties (Roberts and Wink, 1998).

The first report stating the effectiveness of emetine in the treatment of cancer appeared in 1918 (Lewisohn, 1918) and was revived in 1969 (Panettiere and Coltman, 1971). Nowadays, emetine is an important tool in cell biology and *in vitro* pharmacological studies when inhibition of protein biosynthesis is required (Möller and Wink, 2007b).

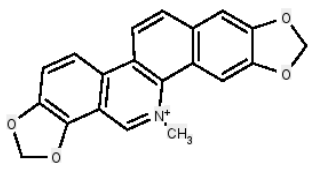


emetine

Emetine is well known to inhibit eukaryotic protein biosynthesis by blocking the elongation process (Grollman, 1968; Grollman and Huang, 1973) and was described to intercalate DNA (Wink, 2003). Like many other protein biosynthesis inhibitors, emetine has the ability to induce apoptosis in human tumour cells (Kochi and Collier, 1993; Bicknell *et al.*, 1994).

3) Sanguinarine

Sanguinarine is from the group of benzo[c]phenanthridine alkaloids. It is extracted from *Sanguinaria canadensis* (Papaveraceae). It has been discovered in milk of lactating cows that were feeding on plants containing this alkaloid (Wink and Wyk, 2008). Sanguinarine is used in toothpastes and mouthwashes (Roberts and Wink, 1998; Wink and Wyk, 2008).



sanguinarine

It has been identified that sanguinarine modulates a number of molecular targets such as: intercalation to DNA, complexation of SH groups in enzymes, inhibition of esterases, Na^+/K^+ ATPase, alanine aminotransferase, human sputum elastase and others. Some of affected organisms are: several *Escherichia* and *Aerobacter* species, *Bacillus anthracis*, *Trichomonas vaginalis*, several *Vibrio* species. It is also active against several oral microbial isolates, such as several *Actinomyces*, *Bacteroides*, and *Propionibacterium* species. Furthermore, sanguinarine has adrenolytic, sympatholytic, anti-inflammatory, cytotoxic, antifungal, and local anesthetic effects (Roberts and Wink, 1998; Wink, 2007; Wink and Wyk, 2008).

1.2 TELOMERE BIOLOGY

1.2.1 Telomere hypothesis

In 1938, a geneticist Müller for the first time named the structure which locates at the end of chromosomes “terminal gene”, afterwards, the name was modified as “telomere” from the Greek terms “telos” (end) and “meros” (part) (Müller, 1938). Müller observed that the ends of irradiated chromosomes of fruit flies (*Drosophila melanogaster*) did not reveal the alteration which often appeared in the other genome, such as deletions or inversions. This finding has been traced in molecular biology and genetics study for decades ever since. It was about two years after Müller found telomeres, Barbara McClintock from the University of Missouri, who worked in corn genetics, claimed that telomeres direct a crucial role in the integrity of chromosomes (McClintock, 1941). However, the telomere sequence and its associated proteins were for the first time identified from *Tetrahymena* by Elizabeth H. Blackburn *et al*. in 1978. In their study, they found that telomere consists of tandem repeat DNA sequences, and it is a highly conserved structure at each end of chromosome (Blackburn and Gall, 1978). From that moment on, telomeres have been cloned and sequenced from diverse organisms, such as ciliates, yeast, plants, and mammals (Table 1). After comparing with the telomeric sequences identified from different organisms, people found that the telomeric DNA sequences are very similar and they are all made up of variable number of tandemly repeated, simple, GC-rich sequences (Blackburn and Challoner, 1984a; Moyzis *et al.*, 1988).

Table 1. Telomere sequence in different organisms

Organism	**telomeric repeat sequence (5'-3')**
Vertebrate	
Homo sapiens	TTAGGG (Blackburn 1991)
Invertebrates	
Cassiopeiae sp.	TTAGGG (Ojimi 2009)
Ampedus sanguineus	TTAGG (Frydrychova 2004)
Caenorhabditis elegans	TTAGGC (Cangiano 1993)
Fungi	
Schizosaccharomyces pombe	G_{2-8}TTAC(A) (Murray 1986)
Saccharomyces castellii	TCTGGG(TG)1-4 (Cohn 1995)
Aspergillus oryzae	TTAGGGTCAACA (Kusumoto 2003)
Neurospora crassa	TTAGGG (Schechtman 1990)
Plants	
Nicotiana tabacum	TTAGGG (Weiss 2002)
Solanum lycopersicum	TT[T/A]GGG (Ganal 1991)
Strombosia pustulata	TTTTAGGG (Teixeira 2005)
Algae	
Cyanidioschyzon merolae	AATGGGGGG (Nozaki 2007)
Chlamydomonas reinhardtii	TTTTAGGG (Petracek 1990)

The end replication problem was first proposed by Watson in 1972. He described that during the process of replication, after the double strands are separated by helicase, the replication on the leading strands can be elongated continuously all the way to the end of template following 5'-3' direction. But on the opposite lagging strand, replication is carried out in segments fashion, called Okazaki fragments. A problem occurs when the lagging strand nears the end of a chromosome. There is no DNA sequence to serve as template for the next Okazaki fragment to fill in the gap between the last Okazaki fragment and the end of a chromosome. Thus, the extreme end of chromosome is not replicated. This incapability may lead to the loss of essential genes eventually on the end of a chromosome. Fortunately, this problem was solved by the existence of telomeres. The lengths of telomere are quite different between species, from approximately 300 - 600 base pairs in yeast (Shampay *et al.*, 1984) to 25 - 150 kilo base pairs in mice (Kipling and Cooke, 1990). Human telomere length is in the range of 10-15 kbp in normal somatic tissues of young persons, 7–8 kbp at old ones and the shortest of 1.5–2 kbp in crisis cells (Greider, 1996; Martens *et al.*, 1998; Meyerson, 2000; Shay and Wright, 2005). Approximately 50-200 bp of telomeres are lost in every cell division due to the end replication problem (Fig 2) (Watson, 1972; Harley *et al.*, 1990). The telomeric DNA does no carry any complex genomic information, but instead a simple, GC-

rich sequence repeated dozens even thousands of times at each end of chromosome. The telomeric DNA can bind specific proteins, which cap the chromosome ends either directly (Horvath *et al.*, 1998) or by inducing a special DNA structure like T-loop (Griffith *et al.*, 1999; Cech, 2000). It has demonstrated such protection involve into several essential biological regulations, protecting chromosomes from recombination, end-to-end fusion, and been recognized as damaged DNA. Telomeres also provide a means for complete replication of chromosomes, contribute to the functional organization of chromosomes within the nucleus, and participate in the regulation of gene expression (McClintock, 1941; Pologe 1988).

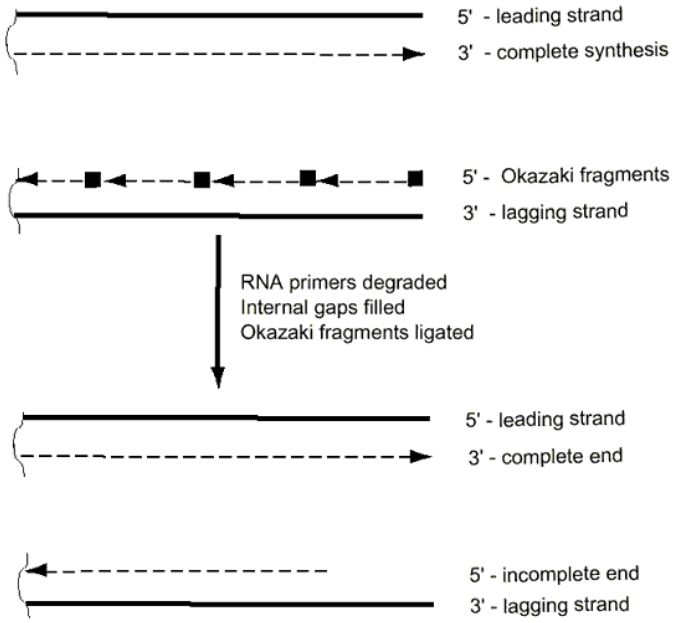


Fig 2. **End replication problem** (Rubin, 2002)

The link between cell viability and telomere length has now been established in a number of different organisms. During cell proliferation, the telomeres get progressively shortened of 50-100 base pairs in each round of replication (Harley *et al.*, 1990). According to Hayflick limit theory (Hayflick, 1965), this shortening in normal cell serves as a molecular clock that controls the replicative capacity of individual cells. After a certain numbers of cell divisions, the telomere length becomes extremely short, and then cells stop dividing and undergo replicative senescence.

1.2.2 Telomere structure and associated regulation

1.2.2.1 T loop

Human telomeres consist of variable number of tandem repeats of the TTAGGG sequence and a group of specific proteins. Electron microscopy analysis of psoralen cross-linked human telomeric DNA revealed abundant large t loops with a size distribution consistent with their telomeric origin. At the 3' end, the G rich overhang invades into the duplex telomeric DNA repeat array to form a D-loop and T-loop structure in vitro (Greider, 1999; Griffith *et al.*, 1999). This structure appears to protect the telomeres from end to end fusion with other chromosomes and from cell cycle checkpoints that would otherwise recognize the telomeres as chromosome breaks requiring repair (Blackburn, 1984b; 1991).

Telomeres are nucleoprotein complexes, consisting of DNA repeat and a series of proteins (Moyzis *et al.*, 1988; Blackburn, 2000). Some associated proteins bind directly onto the T_2AG_3 repeats; and the others perform the regulation via protein-protein interaction (Fig 3).

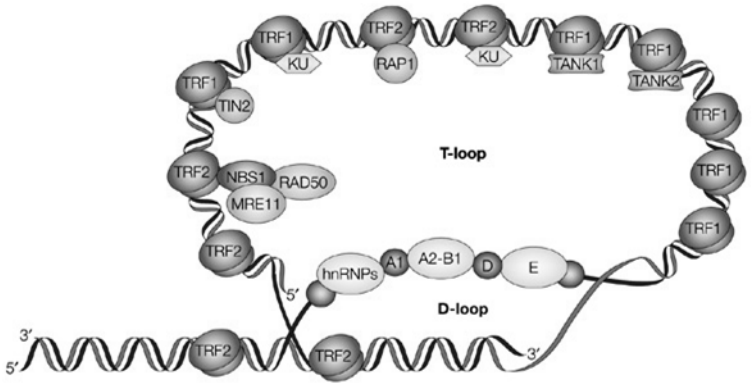


Fig 3. **Telomere T-loop structure with telomere-specific binding proteins**. The single-stranded DNA at the end of the telomere is able to invade and anneal with part of the duplex DNA (D-loop) in the same telomere, with the overall result being a telomere (T)-loop. Several proteins bind directly to telomeric DNA, whereas others are associated with telomere via protein-protein interaction (Neumann and Reddel, 2002).

In human beings, TRF1 and TRF2 proteins specifically bind double stranded telomere sequences via a helix motif (Chong *et al.*, 1995; Bilaud *et al.*, 1996). Both of TRF1 and TRF2 and their associated proteins, such as RAP1p, tankyrase, KU are primarily responsible for stabilizing the complex and forming the T-loop. It has been demonstrated that TRF1 and

TRF2 are involved in regulating telomere length, displaying an inhibitory effect on telomere maintenance *in vivo* (Granger *et al.,* 2002). In some research, the over-expression of TRF1 and TRF2 led to an eventual shortening of telomere in telomerase positive cells (Smogorzewska *et al.,* 2000; Ancelin *et al.,* 2002). Furthermore, results obtained from different studies showed that TRF1 mainly regulates telomere length (van Steensel and de Lange, 1997), whereas TRF2 appears to be particularly abundant at the base of the T-loop and is important for its stabilization and formation (Stansel *et al.*, 2001). The proteins which are involved in telomere regulation *in vivo* are listed (Table 2).

Table 2, Telomerase-/telomere-associated proteins and their interaction (Olaussen *et al*,. 2006)

Factors	Name in human	Effect on telomere	Interactions with
Telomere specific protein			
Telomerase accessory factor	EST1A,EST1B		Telomerase
G-tail binding factors	POT1	Binds T_2AG_3 using OB-fold	TRF1, TRF2, PTOP, TIN2 Tankyrase 1
Duplex	TRF1	Binds telomeres, negative length regulator	POT1, TRF2, PTOP, TIN2 PINX1, TANK1/2, Ku, BLM
	TRF2	Binds telomeres,	POT1, TRF1, hRAP1,
PARP2,		negative length regulator, role in T-loop, chromosome stability	TIN2, MRN, ERCC1/XPF, WRN, BLM, Ku, ATM
Proteins indirectly binding telomere	hRAP1	Length regulator	TRF2, MRN
	TANK1/2	PARP activity, TRF1 ribosylation, positive length regulator	TRF1
	TIN2	Positive length regulator	TRF1, TRF2
	PINX1	Telomerase inhibitor	TRF1, TIN2
Others			
DNA repair proteins	Ku70/Ku86	NHEJ, telomere localization, negative length regulator, telomere capping	Telomerase, TRF1, TRF2
	DNAPKcs	NHEJ, telomere localization. telomere capping	
	Mre11/Rad50/Nbs1	Recombination, NEJ	TRF2
	ERCC1/XPF	NER	TRF2
Checkpoint proteins	ATM	DNA damage signaling	TRF1, TRF2

1.2.2.2 G-quadruplex

The non-coding repeats of guanine-rich DNA as present in the 3'-overhang of human telomeres, have been shown to form tetrastranded DNA structures termed G-quadruplexes.

So far, only two types of intramolecular G-quadruplex, basket- and propeller-type, have been identified in human telomeric sequences in presence of sodium or potassium ions (Wang and Patel, 1993; Parkinson *et al.*, 2002). It has been calculated that the human genome contains as many as 376,000 potential G-quadruplexes forming sequences (Huppert and Balasubramanian, 2005; Todd *et al.*, 2005). These sequences are concentrated in promoter regions (Rawal *et al.*, 2006; Hershman *et al.*, 2008), introns (Huppert and Balasubramanian, 2005), 5' and 3' UTRs (Huppert *et al.*, 2008), and at the ends of eukaryotic chromosomes (Mergny *et al.* 2002; Cuesta *et al.* 2003), suggests that G-quadruplexes may have importance function in vivo. Based on the knowledge that folded G-quadruplex structures are inactive substrates for telomerase (Zahler *et al.*, 1991), some small molecules, for instance the cationic porphyrin TMPyP4 (De Cian *et al.* 2007; Reed *et al.* 2008), the anionic porphyrin N-methyl mesoporphyrin (NMM) (Arthanari *et al.*, 1998), and telomestatin (SOT-095) (De Cian *et al.*, 2007; Reed *et al.*, 2008) have been developed as telomerase inhibitors by stabilizing G-quadruplex-folded structures in the 3' overhangs of telomeric DNA. All these interactions were performed in vitro so far, the development of G-quadruplex-specific antibodies generated by in vitro evolution may provide key tools for target validation (Schaffitzel *et al.*, 2001; Fernando *et al.*, 2008), while the design and synthesis of new high affinity G-quadruplex ligands will provide new drug candidates and molecular probes (Luedtke, 2009).

1.2.3 Telomerase

Telomerase is a cellular ribonucleoprotein complex responsible for synthesizing and adding telomeric repeats onto the 3' end of chromosomes (Blackburn *et al.*, 1989; Le *et al.*, 2000). It is a slow metabolic protein with a half-life over 24 hours (Holt *et al.*, 1996b). The core of the enzyme is composed of two major components: an enzymatic reverse transcriptase, in human called hTERT and an RNA component, in human hTR, which contains a 11-bp sequence (CAAUCCCAAUC) complementary to the telomeric single strand overhang. The telomerase uses its RNA component to serve as a template to synthesize telomeric DNA repeats (TTAGGG)*n* together with the catalytic activity of its protein subunit. The complete telomere elongation reaction includes four events (Fig 4): 1) telomere end recognition, 2) RNA template alignment, 3) elongation and 4) translocation. First, the RNA template recognizes the end of telomeres via an anchor site which locates in hTERT, and then the 11-base

sequence serves as template to synthesis the telomeric DNA. In the end, the complementary strand is completed by DNA polymerase. Each repeat can be achieved via a "slippage" mechanism, during which one repeat is synthesized, the enzyme slips along and repositions itself at the new end of chromosome and repeat the process.

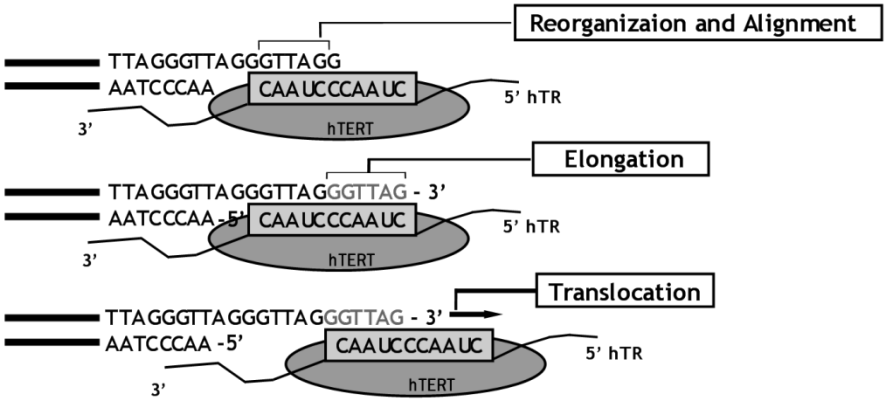


Fig 4. **Model for elongation of telomere by telomerase.** Human hTR consists of 11-basepairs as a template. After recognition of the single strand 3'overhang GTTAGG by telomerase, some nucleotides are hybridized to the CAAUCC sequence in the RNA, and then the sequence GGTTAG is synthesized at a time. Once a repeat is synthesized, the enzyme translocates to a new end and the process is repeated over and over again (Granger *et al.*, 2002).

It was demonstrated that telomerase is actually one of the critical enzymes to make cells immortal; the activity is tightly linked to tumor malignancy, irrespective of the tissue type. Telomerase was detected in 15.5% of pathology positive tissue or adjacent to malignancy; in 29.5% of pre-invasive cancer and in 84% of malignant cancer cells (Kim *et al.*, 1994), whereas is undetectable in most normal somatic cells. Recent research found that the telomerase became detectable when the embryo had reached the 128 to 256-cell blastocyst stage (Wright *et al.*, 1996).

1.2.4 Telomerase complex

1.2.4.1 hTERT

Telomerase reverse transcriptase was first purified from *Euplotes aediculatus* termed as p123 (Lingner *et al.*, 1997). Almost simultaneously, the human catalytic subunit was identified by

four groups (Kilian *et al.*, 1997; Meyerson *et al.*, 1997; Nakamura *et al.*, 1997; Harrington *et al.*, 1997b). They found that the reverse transcriptase motifs are conserved among different organisms (Nakamura *et al.*, 1997). Other recently study showed that the C terminus of hTERT possess more potential functions compared to the other organisms, and this specificity is believed to be the functions unique to higher eukaryotes (Banik *et al.*, 2002).

The hTERT gene had been previously mapped to the distal part of chromosome 5p15.33 (Meyerson *et al.*, 1997; Bryce *et al.*, 2000). Human telomerase catalytic subunit encompasses over 35 kb, with 16 exons and 15 introns (Cong *et al.*, 1999; Wick *et al.*, 1999)(Fig 5). hTERT contains a telomerase-specific T motif and seven other motifs which are highly conserved in reverse transcriptase (Nakamura *et al.*, 1997; Cong *et al.*, 1999). The T motif locates very close to the N terminus of hTERT. Some research revealed that it has a critical role for the enzymatic activity and is responsible for the recognition of the hRT component (Lai *et al.*, 2001). Sequencing analysis revealed that the hTERT promoter is GC-rich, contains several binding sites for different transcription factors (Cong *et al.* 1999), a notable one is myc binding site, which is known to induce hTERT expression (Wang *et al.*, 1998).

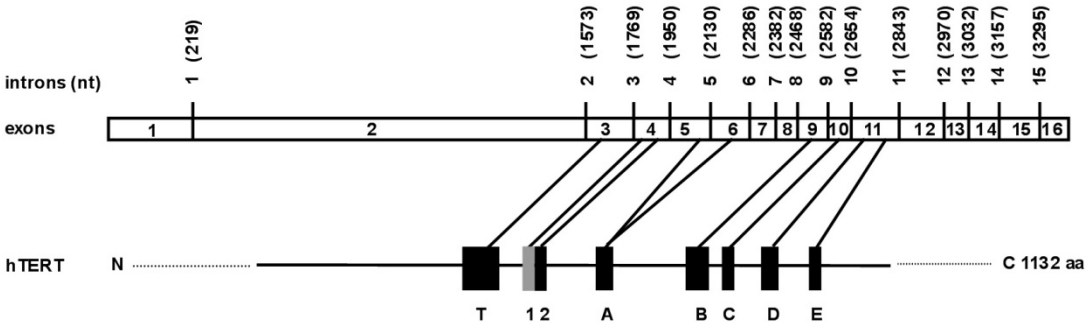


Fig 5. Localization of the exons and introns on the hTERT cDNA: 1, numbers in brackets indicate the extrons; solide cubes in turn indicate the T-motif and other seven motifs (Cong et al. 1999).

An apparent correlation between the presence of hTERT mRNA and telomerase activity has been found (Meyerson *et al.*, 1997; Ducrest *et al.*, 2001). The human catalytic subunit was only detectable in various types of ovarian cancer cells, but not in normal, benign or low potential malignant (LPM) lesion of the ovary (Kyo *et al.*, 1999). Based on these findings, inhibition of the expression of the catalytic subunit TERT has become a major target in anticancer drug development

1.2.4.2 hTR

The RNA component (hTR) serves as a template for telomeric repeat synthesis. In human, hTR contains 451 nucleotides. The 11-basepair template sequence locates between 46 to 53, which is used for hybridization of the end of telomere (Feng *et al.*, 1995). A secondary structure has been identified from different vertebrate species containing four conserved functional elements: CR2/CR3 domain; a CR4/CR5 domain; a box H/ACA domain and CR7 domain (Mitchell *et al.*, 1999; Dragon *et al.*, 2000). The hTR box H/ACA motif has been shown to be essential for hTR accumulation, hTR 3' processing, and telomerase activity in cells (Mitchell *et al.*, 1999; Dragon *et al.*, 2000; Dez *et al.*, 2001), however, it is phylogenetically conserved among vertebrate telomerase RNAs (Chen *et al.*, 2000) but not those of yeasts or ciliates, each of which appear to use a different strategy for accomplishing telomerase RNA accumulation *in vivo* (Seto *et al.*, 1999; Aigner *et al.*, 2000). It has been elucidated that the conserved domains within the hTR molecule are recognition sites for hTR binding proteins. A number of RNA binding proteins, such as hGAR, dyskerin, hNOP10, hNHP2, hStau, L22, hnRNP C1/C1, La, and hTERT, interact with hTR and are involved in hTR stability, maturation, accumulation, and functional assembly of the telomerase ribonucleoprotein complex (Mitchell *et al.*, 1999; Dragon *et al.*, 2000; Ford *et al.*, 2000; Le *et al.*, 2000; Ford *et al.*, 2001; Cong *et al.*, 2002). Two regions within the hTR molecule which locate at nucleotides from 1 to 209 and from 241 to 330 interact independently with the catalytic component hTERT (Mitchell and Collins, 2000).

1.2.5 The regulation of telomerase

It has been documented that among the core components of human telomerase complex, only the catalytic component hTERT seems to be the limiting determinant of telomerase activity. It is transcriptionally repressed in many normal cells and is reactivated or upregulated during immortalization. Whereas hTR is transcribed and accumulated in all human somatic cells and almost all human cell lines (Collins and Mitchell, 2002). The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and accessibility and function of the telomerase ribonucleoprotein on telomeres (Collins and Mitchell, 2002).

1.2.5.1 Transcriptional regulation of hTERT

A number of studies have showed that human telomerase is regulated during development and differentiation, mostly through transcriptional control of hTERT. Evidence showed that the expression of hTERT is a critical step during malignant transformation, the inactivity of telomerase is most frequently correlated with the absence of TERT mRNA (Meyerson *et al.*, 1997). Accordingly, the telomerase activity could be induced in normal cells after introducing the hTERT fragment by reporter gene assays (Bodnar *et al.* 1998; Vaziri and Benchimol, 1998).

Several transcription factors have been identified as the candidate hTERT transcriptional activators, such as c-Myc, and Sp1. Some results have showed that the over-expressed c-Myc transcriptional factor could directly target hTERT gene through the canonical E-box elements (CACGTG), and induced its transcription (Horikawa *et al.*, 1999; Wu *et al.*, 1999; Oh *et al.*, 1999a). The hTERT promoter also contains binding sites for the zinc finger transcription factor Sp1 (Cong *et al.*, 1999; Horikawa *et al.*, 1999; Takakura *et al.*, 1999). Sp1 could dramatically induce the hTERT promoter activity. The direct evidence was obtained by the EMSA analysis in several human cancer cells (Takakura *et al.*, 1999). However, due to the fact that Sp1 is ubiquitous expressed in a wide range of normal cells, the protein by itself is unlikely to be a determinant of hTERT activator during carcinogenic processes (Horikawa and Barrett, 2003). Sp1 may interact with some other proteins to negatively regulate the hTERT transcription in normal cells, and to facilitate its function as an activator in cancer cells.

A series research for the candidate hTERT transcriptional repressors was performed by Oh *et al.* The inhibitory effect on the hTERT promoter activity by the over-expressed WT1 was observed (Oh *et al.*, 1999b). However, WT1 showed a restrict expression in specific tissues (kidneys, gonads and spleen) (Haber and Buckler, 1992) or cell lines (in 293 cells but not in HeLa cells) (Oh *et al.*, 1999b) implying that the transcriptional repression of WT1 on hTERT is tissue-specific. Another identified hTERT transcriptional repressor was an E-box binding factor Mad1 (Oh *et al.*, 2000), which competes with c-Myc for the common interacting partner Max and for the binding E-box element. Some tumor suppressor proteins or pathways

may regulate the hTERT transcription by acting through potential activator. For example, the TGF-β/Smad signaling pathway was known to down-regulate c-Myc expression (Chen *et al.*, 2001; Yagi *et al.*, 2002). The activation or suppression of this pathway in human cancer cells led to a significant reduction or induction of hTERT expression and telomerase activity, respectively (Yang *et al.*, 2001).

It has been demonstrated that hTERT undergoes alternative splicing and several transcripts have been detected in human cells (Kilian *et al.*, 1997). Two deletion sites and four insertion sites in hTERT mRNA were identified (Fig 6) (Kilian *et al.*, 1997; Wick *et al.*, 1999). With all these splicing sites are able to produce numerous combinations, till now only ten hTERT transcripts have been confirmed (Kilian *et al.*, 1997; Hisatomi *et al.*, 2003; Saeboe-Larssen, 2006). The four insertions (indicated with numbers 1 – 4) cause premature translation termination, and two deletions (Fig. 7, as indicated as α and β) cause a 36-bp and 183-pb loss, respectively. Within all of the transcripts are expressed only the full-length hTERT transcript is associated with telomerase activity (Ulaner *et al.*, 1998), whereas the *α* variant was clarified as a dominant negative inhibitor of telomerase activity (Colgin *et al.*, 2000; Yi *et al.*, 2000), and the β deletion played a negative regulatory role on telomerase activity (Lincz *et al.*, 2008).

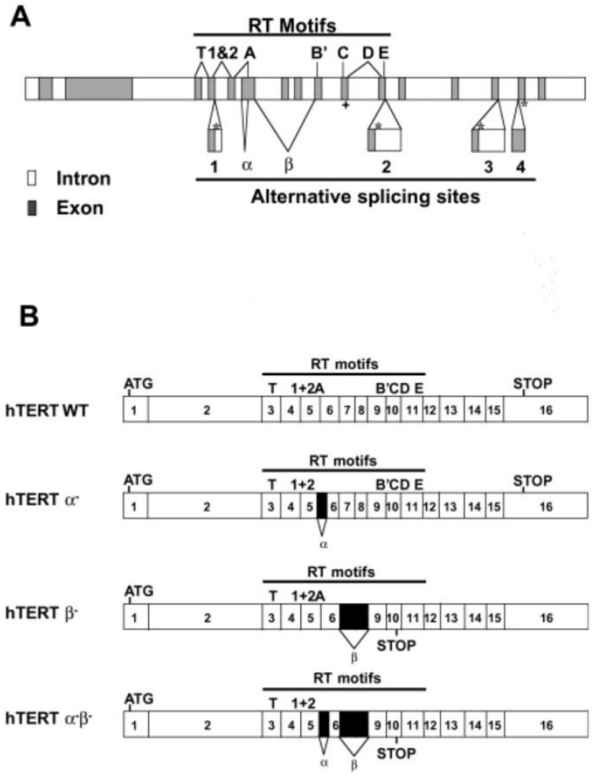


Fig 6. Gene structure of hTERT. A: four insertions and two deletions are indicated with either numbers 1 - 4 or letters α and β. B: Different transcripts generated through alternative splicing. Insertions are not indicated in this figure. Two deletions produce four possible combinations of hTERT alternative splice variants (Yi *et al.*, 2000).

1.2.6 Telomeres and telomerase as targets for anticancer drug development

Cancer is a group of diseases characterized by infinite cell growth and spread of abnormal cells. Cancerous development has been elucidated as a multi-step process with dynamic alternations in the genome (Hanahan and Weinberg, 2000), these alternations are accumulated and eventually drive a progressive transformation of normal cells into a malignant state.

Targeting telomerase: Based on the evidence that over 85% human cancer cells express telomerase activity (Kim *et al.*, 1994), the cancer-associated specificity makes telomerase a superb target for the diagnosis of malignancy and for the development of novel anticancer drugs. Some research has demonstrated that several chemical compounds, like 2-((E)-3-naphtalen-2-yl-but-2-enolylamino)-benzonic acid (BIBR1532) (Damm *et al.*, 2001) or the similar compound 2,3,7-trichloro-5-nitroquinoxaline (TNQX) (Kim *et al.*, 2003), inhibit

telomerase activity, lead to a remarkable cell cycle arrest and initiate apoptosis (Hahn *et al.*, 1999; Damm *et al.*, 2001; Ahmed and Tollefsbol, 2003) and/or premature senescence (Bodnar *et al.* 1998; Damm *et al.* 2001; Gewirtz *et al.* 2008). More and more reports show that targeting telomerase is a valid strategy to combat cancer. However, since telomerase activity could also be detected in germline cells and embryonic cells (Mantell and Greider, 1994), so it seems these normal cells would loose their proliferative capacity during the treatment. Another problem also limits the telomerase-inhibitor usage, the lag period. The lag period highly depends on the initial telomere length in cancer cells, which may require a rather long time to approach critical shortening of telomeres to induce cell cycle arrest (Hahn *et al.*, 1999).

Targeting telomeres: Beside targeting telomerase, the telomeric repeat itself is also a substrate for anticancer agents. As we described earlier, the G-rich single stranded overhang is able to fold back on itself in vitro to form a special G-quadruplex structures. However, the structure of human telomere in vivo is not fully determined, and it remains unclear whether the end of chromosome ends actually form T-loop, G-quadruplexes, or other structures in vivo. Nevertheless, in principle, small molecules are able to stabilize the G-quadruplex structures, presumably by intercalation, and whereby block the access of telomerase during the elongation step (Zahler *et al.*, 1991; Parkinson *et al.*, 2002). To date, a number of G-quadruplex stabilizing ligands have been discovered and tested ever sine the first molecule, 2,6 diamidoanthraquinone was identified (Sun *et al.*, 1997). These G-quadruplex-associated ligands include TMPyP4 (Shi *et al.*, 2001), triazines (12459) (Riou *et al.*, 2002), anionic porphyrins (NMM) (Arthanari *et al.*, 1998) and a natural product telomestatin (Shin-ya *et al.*, 2001). However, in contrast to telomerase, telomeres are present in normal cells as well as in cancer cells, so the risk of cytotoxicity is another issue to overcome for the future agent development.

1.3 CELLULAR SENESCENCE

1.3.1 Introduction

Cellular senescence was originally defined by Hayflick in the early of 1960s (Hayflick, 1965). In his research, he observed that human diploid cell strains, especially fibroblast cells, cease dividing after a certain number of doublings. Via two experiments, he concluded that the

reason why cells stopped dividing was neither the external factor, nor the cumulative period of cell freezing (Larsson, 2005). Thus, he firstly named the phenomenon phase III and later on it was called Hayflick limit, cellular senescence or replicative senescence. The latter two are commonly used in present study. Hayflick's remarkable finding opened up a way to understand, in opposite to normal cell, how cancer cells remain immortal, and therefore, to give the modern research a hint for effective treatments for cancers (Shay and Roninson, 2004).

1.3.2 Replicative senescence

1.3.2.1 The senescent phenotype

Replicative senescence is defined by a set of phenotypic changes including cellular arrest, and the altered morphology. Senescent cells cease dividing but remain metabolically active and produce many secreted factors. The cellular arrest of proliferation accompanied by changes in cell function, such as changes in secretory pathways, expression of protease, extracellular matrix components and inflammatory cytokines (Cristofalo *et al.*, 2004; Shay and Wright, 2005). Senescent cells display a gradual loss of proliferative potential which results in a reduction in cell density (Cristofalo, 1988) and leads to an irreversible growth arrest. However, some research groups have argued that the irreversible status is actually reversible. Recent data has showed that the senescent mouse embryonic fibroblasts (MEFs) could be reversed when p53 expression was inhibited through RNA interference, and such suppression of p53 in senescent MEFs caused a rapid cell cycle re-entry and led to immortalization (Dirac and Bernards, 2003). The senescent cell is mainly characterized by a number of changes in morphology, including cellular enlargement and flattening, the increase in the size of nucleus and nucleoli (Cristofalo and Kritchevsky, 1969; Mitsui and Schneider, 1976; Greenberg *et al.*, 1977) as well as an increase of lysosomes and Golgi (Alvaro *et al.*, 1971; Cristofalo and Kabakjian, 1975). Moreover, the populations of senescent cells reveal an increased number of multinucleated cells (Yanishevsky and Carrano, 1975; Galloway and Buckton, 1978). In addition to the morphological changes, Zhang *et al* has reported that the global changes in gene transcription occurring during replicative

senescence in human fibroblasts and mammary epithelial cells (HMECs), the gene expression perturbations during senescence differ greatly in fibroblasts and HMECs.

1.3.2.2 What drives replicative senescence?

Although a commonly accepted theory has been established, in which the progressively shortened telomeres initiate replicative senescence (Wright and Shay, 1992; Bodnar *et al.*, 1998), as Shay described in his review there still remain some uncertainties about the actual inducing events that 'trigger' senescence (Shay and Wright, 2005).

1.3.2.2.1 The theory of replicative senescence

Telomeres are G-rich structures located at the end of each chromosome. It consists of tandem DNA sequence TTAGGG and associated proteins , such as TRF1, TRF2, TIN2, TPP1, and POT-1, etc, these proteins remain in a complex with telomeric DNA (fig 3) throughout the cell cycle (de Lange, 2005) (More related details have been shown in chapter 2). In normal cells, the progressive telomere shortening occurs alongside each cell division (Allsopp *et al.*, 1995), after a certain number of division, the telomere approaches a critical length, thus, cells stop dividing and enter replicative senescence. The number of cell divisions can be different according to the species, age and genetic background of the donor, the number can be fairly large, for example, 60-80 doublings in some cases (Campisi, 1997). The senescent cells cannot be stimulated to enter the S phase of the cell cycle by any known combination of physiological mitogens. The direct evidences were obtained from in vivo and in vitro show that the extreme shortened telomeres trigger replicative senescence (Smith and Pereira-Smith, 1996; Bodnar *et al.*, 1998; Morales *et al.*, 1999). When the hTERT catalytic subunit was introduced to senescent cells, the cells could bypass senescence and became immortal with ongoing cell division. There are two hypotheses to explain this phenomenon. Firstly, during telomere shortening, the proteins that usually form a complex together with telomeres are no longer able to locate due to the length of the telomere, which consequently initiates a DNA damage response and eventually general senescence (van Steensel *et al.*, 1998; Blackburn, 2000;2001). Secondly, the sequence or the structure of telomere is compromised, which does not particularly imply to the double stranded telomere, but the single stranded telomere. Evidence showed that the loss of 3' G-rich overhangs triggers senescence, 60-85% of the 3'

overhangs are eroded during senescence (Stewart *et al.*, 2003). However, in contrast with this result, Chai *et al* has reported that no loss of single stranded overhang was detected in their research. Results obtained from the conventional nondenaturing hybridization assay showed that the normal human fibroblasts could maintain their overhangs at senescence (Chai *et al.*, 2005).

1.3.2.2.2 Signaling of replicative senescence

Replicative senescence can be initiated in various cell types through different pathways. The most commonly described in ration to senescence are the p16/Rb pathway and the p53/p21 pathway. In this study, we particularly focus our attention on the latter.

The finding has been documented from different groups that replicative senescence is correlated to DNA damage response (Shay and Roninson, 2004; Zhang *et al.*, 2005). Telomeres in senescent cells have typical DNA damage foci, which include nuclear foci of phosphorylated histone H2AX (γH2AX) and its co-localization with DNA repair and DNA damage checkpoint factors such as 53BP1 (Wang *et al.*, 2002), MDC1 (Goldberg *et al.*, 2003) and NBS1 (di Fagagna, 2003; Sedelnikova *et al.*, 2004). γH2AX has gained much attention for its relationship with DNA damage, particularly double strand breaks (DSBs) (Redon *et al.*, 2002; Pilch *et al.*, 2003). The phosphorylated γH2AX forms localized "foci" at the sites of DSBs (Rogakou *et al.*, 1998; Paull *et al.*, 2000) and it is dependent on signaling pathways involving p53, $p21^{WAF1/CIP1}$ but not $p16^{INK4a}$ (Herbig *et al.*, 2004).

p53 encoded by the tumor suppressor gene has been reported to act as an integrator of various cellular events, such as cell cycle arrest, apoptosis, differentiation, and lately described senescence. The in vitro result suggested that p53 is the most noticeable regulator in cellular senescence (Sugrue *et al.*, 1997). p53 is presumed having potential roles for recognizing dysfunctional telomeres and maintaining telomere complex integrity due to the observation that p53 binds to the single stranded telomeric overhangs, and thereby, cooperates with TRF2 in the formation of the T-loop (Stansel *et al.*, 2002). Although it has been investigated that the senescence response to telomere dysfunction is reversible and is maintained primarily by p53 (Beausejour *et al.*, 2003), the precise roles of this process are not fully understood. p53 is postulated to treat dysfunctional telomeres as damaged DNA,

which in turn activates p53. p53 can be activated through different signals, some of them via post-translational modification like acetylation or phosphorylation (Shieh *et al.*, 1997; Kapoor and Lozano, 1998; Sakaguchi *et al.*, 1998; Webley *et al.*, 2000). The p53 gene performs a critical regulation in the cell cycle (Levine, 1997; Gire and Wynford-Thomas, 1998), when a genotoxic stress occurs, the increased level of p53 protein stops the cell cycle either at G_1 or G_2 (Levine, 1997). Cell growth arrest in senescence is tightly associated with switching on the transcriptional function of p53, which was assessed by using reporter constructs and by DNA binding assays (Bond *et al.*, 1996). A role of p53 in replicative senescence emerged lately (Gewirtz *et al.*, 2008), and was enforced by the observation that both antisense p53 and papillomavirus protein E6, which mediates p53 degradation, lead to an extension of life span in human diploid fibroblasts (Scheffner *et al.*, 1990; Hara *et al.*, 1991; Shay *et al.*, 1991).

Table 3. Induction of cell fate in human cancer cells following chemotherapy or radiation (Gewirtz *et al*,. 2008)

Cell line	p53 status	Modality	Cell fate
MCF-7	+	AdR	Senescence + MC[a]
	+	SN-38, IR	Senescence
MCF-7 hTERT	+	AdR	Senescence
MCF-7 HPV-16 E6	-	AdR	Apoptosis >> senescence
	-	IR	Apoptosis >> senescence
MDA-MB231	-	AdR	Apoptosis >> senescence
	-	IR	Apoptosis >> senescence
HCT116	+	AdR	Senescence + MC
	+	VP-16, SN-38, camptothecin, cisplatin	Senescence
HCT116 p53-/-	-	AdR, VP-16	Apoptosis + MC >> senescence
	-	SN-38, cisplatin	Apoptosis >> senescence
	-	Camptothecin	Apoptosis
HCT116 p21-/-	+	AdR, VP-16, SN-38, IR, Camptothecin	Apoptosis
	+	Cisplatin	Apoptosis >> senescence
H1299	-	VP-16	Senescence
	-	IR	Apoptosis
H1299 + p53	+	IR	Senescence
Glioblastoma	+	SN-38, IR	Senescence
Glioblastoma p53-/-	-	IR	Apoptosis

a MC: mitotic catastrophe.

Once activated, p53 induces many other transcriptional factors involved in cell cycle arrest or apoptosis (Giaccia and Kastan, 1998; Zhao *et al.*, 2000), one of them is p21. p21 is a cyclin-dependent kinase (CDK) inhibitor, which inhibits CDK activity throughout all phases of cell cycle by directly binding with multiple cyclins and CDKs and, therefore, inhibiting CDK

activity in vivo and in vitro (Harper *et al.*, 1993; Xiong *et al.*, 1993). The growth arrest which is caused by p53 is believed to occur through transcriptional activation of the cyclin-dependent kinase inhibitor p21 (fig 7) (Eldeiry *et al.*, 1993; Harper *et al.*, 1993; Agarwal *et al.*, 1995). During p53-dependent cell proliferation repression, p21 is important for coordinating cell cycle progression, DNA replication, and repair of damaged DNA. Recent studies have suggested that p21 mainly inhibits CDK2/cyclin-E (Sherr and McCormick, 2002) and lesser effect on CDK4/cyclin-D (Giaccia and Kastan, 1998). Although it was predicted that p21 inhibits Cdk2 activity by a similar mechanism of p27 (Zhu *et al.*, 2005), in which p27 inhibits CDKs by inserting into the catalytic cleft (Jeffrey *et al.*, 1995), the actual interactive pattern between p21 and Cdk2 remains unclear. In telomere-associated senescence, p53 activates p21 gene expression in response to DNA-damaging stress, and the increased level of p21 protein then binds CDKs complexes, resulting in cell cycle arrest (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). An important role of p21 for the induction of senescence in human cells has been revealed from different researches. Some data showed that p21 was able to induce senescence in a p53-independent manner (Fang *et al.*, 1999; Wang *et al.*, 1999); and the other result showed that p21-deficient human fibroblast cells could bypass senescence (Brown *et al.*, 1997; Wei *et al.*, 2001).

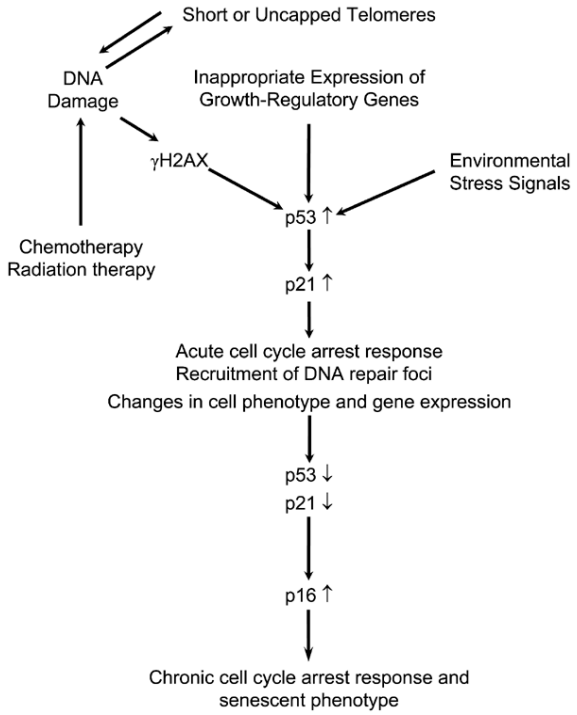


Fig 7. Induction of senescence in normal cells (Shay and Roninson, 2004).

1.3.3 Premature senescence

In addition to replicative senescence which is caused by the progressive telomere shortening, growing evidence showed that telomere dysfunction leads to an "uncapping" situation by several stimuli in normal and malignant cells (Serrano *et al.*, 1997; Robles and Adami, 1998; Serrano and Blasco, 2001; Ramirez *et al.*, 2003), such stimuli include UV- and γ-irradiation (Gorbunova *et al.*, 2002), transformaing growth factor (TGF-β) (Katakura *et al.*, 1999; Untergasser *et al.*, 2003), hydrogen peroxide (Duan *et al.*, 2005) and some chemotherapeutic agents (Chang *et al.*, 1999a; Christov *et al.*, 2003). Many anticancer agents induce apoptotic cell death through activating DNA damage signaling pathways. However, it was lately argued that in some cases cells do not die but instead undergo a senescence-like cell cycle arrest (Chang *et al.*, 1999b; Elmore *et al.*, 2002; Zhao *et al.*, 2004). Mounted data suggest that such cellular stimuli can initiate the change of telomeric DNA structure or sequence (Crabbe *et al.*, 2004), mutation of telomere-associated proteins (Machwe *et al.*, 2004; Yang *et al.*, 2005), which in turn trigger premature senescence (Serrano *et al.*, 1997; Robles and Adami, 1998; Naka *et al.*, 2004; Shay and Roninson, 2004). Furthermore, the uncapped state leads to a rapid cell cycle arrest, at the meantime accompanied with many of the same morphological and biochemical features for replicative senescence. The growth arrest which is caused by cellular stimuli is in fact similar to replicative senescence, and also fails to react to mitogens stimulation. Moreover, premature senescent cells also remain metabolic and synthetic activity (Serrano *et al.*, 1997; Drayton and Peters, 2002; Shay and Roninson, 2004).

The response of cells to replicative senescence versus premature senescence may or may not be similar, but in both cases, the compromising of telomere function leads to cell growth arrest or cell death, and a common possibility, DNA damage response, may be involved in both issues.

1.3.4 Cellular senescence and cancer

Shay and Campisi have pointed out that, at least in human cells, replicative senescence is a powerful tumour suppressive mechanism (Campisi, 1997; Shay and Roninson, 2004).

An important distinction among all kinds of organisms is to have renewable tissues that are essential for viability, and these renewable tissues have evolutionarily conserved defense

mechanisms that guard against unrestrained proliferation (Campisi, 2003; Shay and Roninson, 2004). A progressive accumulation of damage must take place alongside the cell division; such damage can be generated through epigenetic or genetic pathway. Cancer cells must accumulate many mutations before acquiring malignant characteristics. Therefore, limiting the number of available cell divisions would prevent pre-malignant cells from dividing after accumulating only a few mutations, and thus block their progression (Campisi, 2003; Shay and Roninson, 2004).

Cellular senescence is also believed, at least among vertebrates, to be a evolutionary conserved process (Campisi, 2003). The senescence phenotype combined with cell growth arrest were observed in different species under certain conditions (Campisi, 2001). Moreover, senescence was also identified in non-telomere associated cases (Margolis and Spradling, 1995; Jazwinski, 1996). People found that Yeast or *Drosophila* ovarian stem cells could also undergo senescence after several times of division, but no telomere shortening was observed in senescent cells.

Evidence showed that most cell lines are senescence inducible in counteracting some chemotherapy treatment (Chang *et al.*, 1999a) and this result was confirmed by the in vivo response to chemotherapy in mice (Chang *et al.*, 1999a; Schmitt *et al.*, 2002). It was speculated that cancer cells acquire mutations through several common signaling pathways (Yeager, 1998; Hanahan and Weinberg, 2000), these mutations tend to inactivate either the p53 or RB pathways or their upstream positive regulators, which in turn prevent the senescence response. This hypothesis was afterwards confirmed from different groups. When manipulating the expression of p53 and Rb in vitro, it causes cells either to ignore senescence-inducing signals (Hara *et al.*, 1991; Shay *et al.*, 1991; Gire and Wynford-Thomas, 1998) or to arrest growth with a senescent phenotype (Sugrue *et al.*, 1997; Bringold and Serrano, 2000; Lundberg *et al.*, 2000; Itahana, 2001). However, the senescence-promoting and senescence-suppressing factors in cancer cells exist in a dynamic equivalent pattern, which can be shifted in favor of senescence by a variety of cellular stimuli, and thereby to inhibit cancer cells growth (Bertram *et al.*, 1999; Goodwin *et al.*, 2000; Wells *et al.*, 2000). Taken together, the enhancement of the program of senescence in cancer cells provides a biologically approach to cancer therapy.

1.4 AIM OF THE WORK

Plants produce plenty of secondary metabolites (SM), the alkaloids are one of the most diverse groups of SM found in living organisms and have a series of structure type. Many alkaloids have been identified possessing strong toxic properties towards animals and human. During the past decades, more attention has been drawn on their anticancer potencies. A number of alkaloids have been used as anticancer drug over 40 years.

In this study, three alkaloids harmine, emetine, and sanguinarine were selected. Data obtained from in vitro studies showed that these alkaloids commonly interact with DNA, preferentially with G-quadruplexes in a manner of intercalation. Human telomere has been characterized able to form two types of G-quadruplex. However, little direct evidence of in vivo study is available so far. Therefore, we introduced these compounds into different human cancer cells in order to determine their anti-cancer properties and to investigate the effect on human telomerase and the associated pathways. We particularly focused our attention on the alkaloid harmine.

The aim of this work can be summarized as

1. To determine the anti-proliferative and cytotoxic properties of harmine, emetine, and sanguinarine in human breast cancer MCF-7 cell line and human cervical cancer HeLa, and SiHa cell lines.
2. To investigate the inhibitory effect of harmine, emetine, and sanguinarine on human telomerase.
3. To investigate and clarify the potential pathway in MCF-7 and HeLa cells after the treatment of harmine.

2 MATERIAL AND METHODS

2.1 CELLS

In order to carry out this project, three different carcinoma cell lines- HeLa, SiHa and MCF-7- were used. Hela and SiHa cells are human cervical cancer cells. HeLa cells were routinely cultured in our lab; SiHa cells were kindly offered by Dr. Thomas Hofmann (German Cancer Research Center, DKFZ). MCF-7 is human breast adenocarcinoma cell line which was kindly provided by Prof. Dr. Stefan Wölfl.

2.2 INSTRUMENTS

Instrument	**Company**
Centrifuge Hermle ZK 364	M & S Laborgeraete GmbH, Wiesloch
Centrifuge J2-21, Rotor JA-14	Beckmann, Muenchen
Centrifuge Megafuge 1.0R	Heraeus Sepatech, Wiesloch
CO_2 incubator B5060 EK/CO_2	W. C. Heraeus GmbH, Hanau
Experion™ automated electrophoresis station	Biorad, Muenchen, Germany
Experion™ priming station	Biorad, Muenchen, Germany
Experion™ vortex station	Biorad, Muenchen, Germany
High pressure steriliser CertoClav	KELOmat – Sterilizer Division, Australia
Hoefer TM SE600 Electrophoresis chamber	Amersham Biosciences, Freiburg
LightCycler Instrument	Roche Biosciences GmbH, Penzberg
Lightmicroscope	Nikon Cooperation, Tokyo, Japan
Liquid Nitrogen Liquide GT35	AIR LIQUIDE GmbH, Duesseldorf
Microplate reader	Tecan Safire2, Crailsheim, Germany
Multistep - Pipette	Eppendorf Deutschland GmbH, Wesseling-Benzdorf
NanoDrop 2000	Thermo Fisher Scientific Inc, USA
Neubauer chamber	Neolab®, Heidelberg
Thermocycler	Biometra GmbH, Goettingen
Pipette	Eppendorf Deutschland GmbH, Wesseling-Benzdorf
Pipettus®	KG, Eberstadt
Precisionbalance Sartorius Basic	Sartorius AG, Goettingen
Spectrophotometer	Eppendorf
UV – Transilluminator	Biogene 6.02 processing system Heidolph Instruments GmbH & Co.KG,
Vortex Heidolph Relax top	Schwabach
Waterbath Julabo P	Julabo Labortechnik GmbH, Seelbach

2.3 LABORATORY MATERIALS

Material	Company
Cellculture flasks Cellstar® 25 cm^2	Greiner bio-one GmbH, Frickenhausen
Cellculture plates Cellstar® (96-well, 24-well, 6-well)	Greiner bio-one GmbH, Frickenhausen
Cryotubes	Greiner bio-one GmbH, Frickenhausen
Distritip multi step syringe (Gilson 12.5 ml, 1.25 ml)	Abimed, Langenfeld
Eppendorf safe lock reaction tubes (2 ml, 1.5 ml, 0.2 ml, 0.1 ml)	Eppendorf AG, Hamburg
MultiGuard™ Barrier Tips (10 µl, 100 µl, 1000 µl)	Sorenson BioScience, Inc
LightCycler capillaries	Roche diagnostics GmbH, Penzberg
Syringe Driven Filter Unit (0.22 µm, 0.45 µm)	Millipore Corporation, U.S.A
Pipettes (serological, sterile, 5 ml, 10 ml, 25ml)	Greiner bio-one GmbH, Frickenhausen
Steritop™ & Stericup® , 500 ml	Millipore Corporation, U.S.A

2.4 KITS

Kit	Company
Absolute ™ QPCR SYBR® green capillary mixes	Abgene House, Surrey, UK
RNeasy Mini kit	QIAGEN, Hilden, Germany
RNase-free DNA set	QIAGEN, Hilden, Germany
ImProm-II™ Reverse Transcription System	Promega, Corporation, Madison, USA
Taq polymerase	Bioron GmbH, Ludwigshafen
SuperSignal West Dura Extended Duration Substrate	Thermo Scientific,

2.5 BUFFER AND SOLUTION

All solutions and buffers were made in sterilized water, if not otherwise indicated.

Phosphate Buffered Saline (PBS)

NaCl	140 mM
KCl	13.4 mM
Na_2HP_4	0.007 mM
HCl	adjust to pH 7.4

Lysis buffer for TRAP assay (DEPC water)

Tris-HCl, pH 7.5	10 mM
$MgCl_2$	1 mM
EGTA	1 mM
CHAPS	0.5%
Glycerol	10%

10 × TRAP buffer (made in DEPC water)

Tris-HCl, pH 8.3	200 mM
$MgCl_2$	15 mM
KCl	680 mM
Tween 20	0.5 %

12.5% Arcylamide Gel solution (400 ml)

40% Acrylamide (19:1)	125 ml
5 × TBE buffer	40 ml
ddH_2O	235 ml

PAGE/0.5 × TBE gel (40 ml)

12.5% Acrylamide solution	12.5 ml
10% APS	400 µl
TEMED	40 µl
5 × TBE buffer	27.5 ml

5 × TBE buffer (pH 8.3)

Tris Base	445 mM
Boric Acid	445 mM
EDTA (pH 8.0)	10 mM

Cell Fixative solution (made in PBS)

2% (v/v) Formaldehyde
0.2% (w/v) Glutaraldehyde

PI staining solution (made in PBS)

PI	50 µg/ml
RNase	0.1 mg/ml
Triton X-100	0.05%

X-Gal stock solution (10 ml)

X-Gal	200 mg
DMF	10 ml

Store at -20 ºC in aliquots

SA-β-Galactosidase staining solution

$K_3[Fe(CN)_6]$	5 mM
$K_4[Fe(CN)_6]$	5 mM
$MgCl_2$	2 mM
NaCl	150 mM
Citric acid/PBS, pH 6.0	40 mM
X-Gal	1mg/ml (add freshly)

NP40 Buffer (for Western Blot)

Tris HCl pH 7.4	20 mM
NaCl	150 mM
EDTA	5 mM
NaF	25 mM
NP40	1%
Glycerol	10%

Bradford reagent

Coomassie Brilliant Blue G-250	100 mg
95% Ethanol	50 ml
85% (w/v) Phosphoric acid	100 ml

Dilute into 1 liter of distilled water when the dye has completely dissolved and filter with Whatman paper

SDS – PAGE (12%)

Acrylamide mix	40%
Tris-HCl pH 8.8 (1.5 M)	375 mM
SDS	0.10%
APS	0.10%
TEMED	0.04%

2 × Sample buffer

Tris-HCl, pH 6.8	130 mM
SDS	6%
Glycerol	20%
β-mercaptoethanol	10%
Bromophenol blue	0.1%

Running buffer

Glycin	194 mM
SDS	0.1%
Tris	33 mM

TAE buffer

Tris	40 mM
EDTA	1 mM
Acetic acid	adjust to pH 8.8

Transfer buffer

Tris-base	33 mM
Glycine	192 mM
Methanol	20%

Blocking buffer (made in TBS-T buffer)

Non-fat-milk	5%
Tween20	0.05%

Store at 4 ºC to avoid bacteria contamination

10 × TBS-T buffer

Tris, pH 8.0	100 mM
NaCl	1.5 M
Tween20	0.1% (as final concentration v/v)

3. METHODS

3.1 Cell culture

3.1.1 Maintenance of cell cultures

All media and solutions which were required in cell culture are listed in table 3. Cells were cultured in DMEM medium containing 10% FBS, 1% 20 mM glutamine, cells were incubated at 37 ºC of 5% CO_2 and passaged every 3 or 4 days. During cell subculture, medium was discarded and 3 ml trypsin/EDTA solution was added into 75-ml flask, returned the flask back to the incubator and incubated for about 3 min. The digestion was stopped by adding 5 ml fresh cell culture medium, the cell solution was pipetted up and down several times in order to separate the cell clumps into single cells, and then ¼ volume of the cell suspension was kept culturing by adding another certain amount of fresh medium. Cell culture medium was replaced during cell culturing only if necessarily.

3.1.2 Cell cryopreservation

To prepare frozen stock cultures, cells were harvested and the cell suspension was centrifuged at 2000 rpm for 10 min. The cell pellet was then resuspended in pre-cooled fresh culture medium containing 10% FBS and 10% DMSO, pipetted gently up and down to separate cell clumps. The density of cell solution was conducted in a counting chamber, and then about 1×10^6 cells were transferred into Cryo-tubes. Cell cryopreservation was performed by the protocol that cell solution was kept at 4 ºC for 30 min and – 20 ºC for 2 h, then the frozen cells were stored in liquid nitrogen.

Table 4. Media and solutions for cell culture

Cell culture media and solution	
DMEM	Dulbeccos's Modified Eagle's Medium (Invitrogen, USA)
FBS	Heat-inactived at 56 ºC 30 min (PAA Laboratories GmbH, Germany)
Freezing medium	DMEM containing 20% FBS, 8% DMSO
Trypsin/EDTA	0.5% trypsin/EDTA

3.2 CELL CYTOTOXICITY ASSAY (MTT ASSAY)

In this study, the MTT assay was taken to assess the cytotoxicity of alkaloids on human cancer cell proliferation. MTT assay was first described by Mosmann (Mosmann, 1983). The method is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave the tetrazolium rings of the pale yellow MTT, and form purple formazan crystals which are impermeable to cell membranes. The crystals can be solubilized by detergents. The number of living cells is directly proportional to the level of the formed formazan, which can be quantified photometrically.

3.2.1 Cell Preparation

Cells were placed into 96-well plates and incubated at 37 ºC overnight; cells were then treated with alkaloids in various concentrations. Each concentration was repeated in triplicate manner. Medium with or without compounds was replaced every 24 hours with fresh medium containing the same concentrations of compounds. Cells were then grown in the continuous presence of drugs up to different time points (24 h, 48 h, 72 h and 96 h). After treatment, cells were collected for either cytotoxicity assay or cell proliferation assay. The experiments were repeated three times independently.

3.2.2 Preparation of harmine, emetine and sanguinarine stock solutions

Harmine ($C_{13}H_{12}N_2O$; MW 212.25), emetine ($C_{29}H_{40}N_2O_4$; MW 480.65) and sanguinarine ($C_{20}H_{14}NO_4$; MW 332.34) were purchased from Sigma Aldrich Company. These compounds were dissolved either in DMSO or in water according to the user instruction, respectively. All stock solutions were prepared as stock concentration of 100 mM, and stored at -20 ºC. The pH values of these solutions were determined after mixing with DMEM medium, each of them displayed the pH value between 7 and 8.

3.2.3 Absorbance measurement

After the treatment with alkaloids, the cell culture medium was removed gently, MTT stock solution was added to a final concentration of 0.5 µg/ml, and incubated at 37 ºC for about 2 to 4 hours until the purple formazan crystals had formed clearly. The MTT solution was discarded carefully, formazan crystal was dissolved in 100 µl DMSO containing 10% SDS and 1% acetic acid. The plates were mounted on top of a shaker and were shaken for 10 min

until the formazan crystals were dissolved completely, the concentration was determined at an optical density at 570 nm in Safire2 Microplate reader. Experiments were repeated three times independently.

3.3 TELOMERASE ACTIVITY ASSAY

The polymerase chain reaction (PCR) based telomeric repeat amplification protocol (TRAP) was originally developed by Kim *et al* (Kim, Piatyszek et al., 1994). It is applied specifically to detect telomerase activity.

Principle (Fig 7): the assay measures enzymatic activity of telomerase. The TS primer acts as a substrate for telomerase-mediated addition of TTAGGG repeats. Addition of the repeats occurs at 25°C, after which PCR is used to amplify the extended products. The TS primer serves as forward primer while CX is the reverse primer for PCR. The products are then separated by electrophoresis on a 12.5% polyacrylamide gel, which is then stained with Sybergreen (Molecular Probes) to visualize the 6 bp ladder. TS primer: AAT CCG TCG AGC AGA GTT, CX primer: CCC TTA CCC TTA CCC TTA CCC TAA. 0.5 µg protein extract is analyzed for each sample. An internal control (IC) is included in TRAP assay, which denotes a 36-bp band that serves to normalize sample to sample variation. IC is unrelated to telomerase activity and is not included in the protein quantitation.

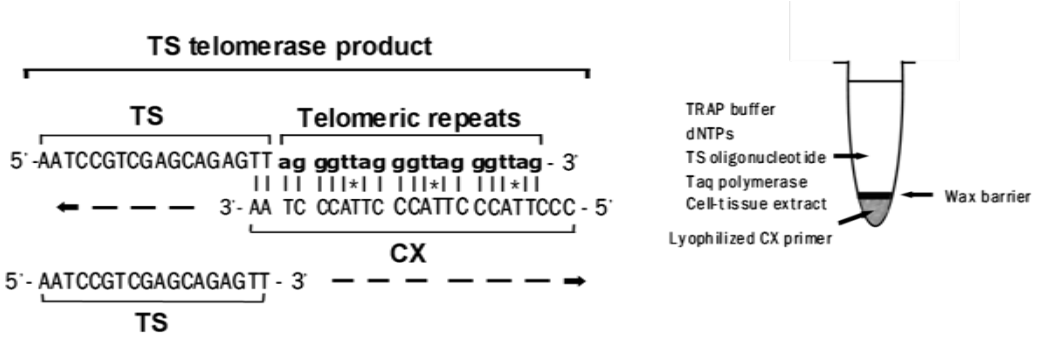


Fig 8. TRAP assay protocol (Kim, Piatyszek et al., 1994)**.**

3.3.1 Protein extract preparation and quantitation for TRAP assay

Cell samples were prepared in 24 well plates and exposed to different compounds, and then the protein extracts were prepared. The cell culture medium was removed and the cells were washed twice in pre-cooled PBS, then the plates were placed on ice and 100 µl of CHAPS

lysis buffer was directly added into each well. The cell lysate was pipetted up and down every 10 min with nuclease-free tips in order to ensure cells were lysed completely, and then centrifuged 12,000 rpm for 30 min at 4 ºC, the supernatants were then transferred into new DEPC treated Eppendorf tubes. The protein concentrations were determined by Bradford assay. In acidic environment, protein binds to coomassie dye, which induces a spectral shift from brown form of the dye. The difference between these two forms is greatest at 595 nm, so it is taken as optimal wavelength for protein-coomassie dye complex measurement. A standard curve was developed by a series of BSA solution in increased concentrations, ranging from 2.5 to 20 µg/ml. The measurement was conducted by spectrophotometry at 595 nm. All steps were carried out on ice in order to avoid protein degradation.

3.3.2 Telomeric Repeat Amplification Protocol (TRAP)

TRAP reaction was performed according to the protocol of Kim *et al* (Kim, Piatyszek et al., 1994). All solutions and buffers were self-made and prepared in DEPC water. 0.5 µg of cell extract was analyzed. CHAPS lysis buffer was included as negative control.

3.3.3 The separation of TRAP productions and quantitation of telomerase activity

TRAP products were separated in non-denaturing 12.5% acrylamide gels. The gels were stained in SYBR green containing solution and visualized under UV – Transilluminator. The intensity of each ladder was then detected and calculated by the software ImageJ (National Institutes of Health, Maryland). The relative telomerase activity (RTA) was conducted by taking the values to the formula (Betts and King, 1999):

$$RTA = \frac{(s-b)/\ ic_s}{(pc-b)/ic_{pc}} \times 100$$

s: Intensity of sample
pc: Intensity of positive control
b: Intensity of background
ic: Intensity of internal control

The values are expressed as % of the control sample.

3.4 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT- PCR) AND RELATIVE QUANTIFICATION-POLYMERASE CHAIN REACTION (Real- Time PCR)

3.4.1 Total RNA isolation, qualification and quantitation

Cell samples were prepared in 6 well plates. After a certain time of treatment, the total RNA was isolated by using RNeasy Mini-Kit (QIAGEN) according to the manufacture's instruction. The RNA pellets were then dissolved in nuclease-free water and stored at -80 ºC until use. The concentrations were determined by a NanoDrop 2000 spectrophotometer. Values of A_{260} were taken for the calculation of total RNA amount following the fomular of 1 A_{260} unit is equivalent to 40 µg/ml RNA. The ratio of A_{260}/A_{280} was taken into account to provide the purity of the RNA samples. Values between 1.8 ~ 2.0 were acceptable.

3.4.2 Reverse transcription

1 µg total RNA was reverse transcripted into cDNA with the ImProm-II™ Reverse Transcription System (Promega) including 0.5 µg random primer, 0.5 mM dNTPs, 5 units ImProm-II reverse transcriptase, 20 units RNasin inhibitor. The reverse transcription was carried out in a 20 µl reaction. The extension was performed at 25 ºC 5 min, 42 ºC 60 min and 70 ºC 15 min.

3.4.3 Polymerase Chain Reaction (PCR)

1 µl of cDNA solution was analyzed by PCR, containing 0.2 mM dNTPs, 0.5 µM of each primer; 2.5 units *Taq* polymerase. In PCR analysis, β-actin was included as housekeeping gene to normalize and standardize the expression of target genes. The overall hTERT gene was amplified with the primers of LT5 and LT6; the hTERT alternative splicing variants were amplified with the primers hTERT-2026F and hTERT-2482V; p21, p53 and CDK2 genes were also analyzed, all primer sequences and PCR protocols are listed in table 5.

Table 5. Primers for reverse transcription-PCR (RT-PCR)

gene	primer sequence (5'-3')	Amplification condition
β - actin (for) β - actin (rev)	CCTGGCACCCAGCACAAT GGGCCGGACTCGTCATAC	94 °C 15 s 60 °C 15 s 20 cycles 72 °C 30 s
LT5 LT6	CGGAAGAGTGTCTGGAGCAA GGATGAAGCGGAGTCTGGA	94 °C 45 s 60 °C 45 s 33 cycles 72 °C 90 s
hTERT-2026F hTERT-2482V	GCCTCAGCTGTACTTTGTCAA CGCAAACAGCTTGTTCTCCATGTC	94 °C 15 s 60 °C 15 s 35 cycles 72 °C 30 s
p21 (for) p21 (rev)	TTTCTCTCGGCTCCCCATGT GCTGTATATTCAGCATTGTGGG	94 °C 15 s 60 °C 15 s 20 cycles 72 °C 30 s
Cdk2 (for) Cdk2 (rev)	CCTCCTGGGCTGCAAATA CAGAATCTCCAGGGAATAGGG	94 °C 15 s 60 °C 15 s 22 cycles 72 °C 30 s
p53 (for) p53 (rev)	TGCGTGTGGAGTATTTGGATG TGGTACAGTCAGAGCCAACCAG	94 °C 15 s 60 °C 15 s 20 cycles 72 °C 30 s

All amplifications were preceded by an initial heating to 95 °C for 2 min, and the extension at 72 °C for 10 min in the end.

3.4.4 Agarose gel electrophoresis

PCR products were subjected to electrophoresis in agarose/TAE gels. 5 µl of each PCR product was analyzed. The PCR products of β-actin, overall hTERT, p21, and CDK2 were loaded onto 1.2% agarose gel and allowed to run at 80 V for 15 min. The product of hTERT alternative splicing variants was loaded onto 2% agarose gel and allowed to run at 40 V for 2.5 h in order to separate the variants bands properly. DNA bands were then visualized with ethidium bromide in the UV – transilluminator. Intensity of each band was determined by ImageJ software.

3.4.5 Relative quantification PCR (Real-Time PCR)

Real-Time PCR technology is a nowadays commonly used analysis method, which has been adapted to perform quantitative RT-PCR (Winer, Jung et al., 1999), and to quantify the amount of the interesting transcript. Compared to the PCR which is conducted by the normal standard protocol, the Real-time PCR is more efficient and accurate. Relative quantification

determines the changes in steady-state mRNA levels of a target gene across multiple samples and expresses it relative to the levels of an untreated control. The reference gene is often a housekeeping gene and can be co-amplified in the same tube in a multiplex assay or can be amplified in a separate tube. SYBER Green provides the simplest and most economical format for detecting and quantitating PCR products in real time PCR. Increasing the temperature leads to the melting of the double strands of DNA, and thereby decreasing Syber green fluorescence. The melting curve is a plot of fluorescence versus increasing temperature.

1 µl cDNA solution was applied in 10 µl PCR reaction in capillaries, containing 1 × SYBR Green Master Mix (ABgene), 0.3 µM of each primer. β-actin was involved as housekeeping gene, a no-template control was also included in each setup as negative control. The PCR reaction was performed in LightCycler3 (Roche).The initial denaturation at 95 °C 10 min, followed with 45 cycles: 95 °C 10 sec; 60 °C 10 sec. Then all crossingpoint (cp) values were assessed by using REST soft relative to the expression of housekeeping gene of β-actin. The primers which were used in Real Time PCR are listed in table 5.

Table 6. Primers for relative quantification PCR

gene	primer sequence (5'-3')
β - actin (for)	CCTGGCACCCAGCACAAT
β - actin (rev)	GGGCCGGACTCGTCATAC
2007hTERT-f	ACGGCGACATGGAGAACAA
2007hTERT-v	CACTGTCTTCCGCAAGTTCAC
p21 (for)	TTTCTCTCGGCTCCCCATGT
p21 (rev)	GCTGTATATTCAGCATTGTGGG
Cdk2 (for)	CCTCCTGGGCTGCAAATA
Cdk2 (rev)	CAGAATCTCCAGGGAATAGGG
p53 (for)	TGCGTGTGGAGTATTTGGATG
p53 (rev)	TGGTACAGTCAGAGCCAACCAG

3.5 PROTEIN ANALYSIS

3.5.1 Preparation and quantitation of protein extacts

The preparations and quantitation of protein for Western blot analysis please see 4.3.1, but The lysis buffer was replaced by NP40 lysis buffer.

3.5.2 SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used technique to separate proteins according to their molecular weights. Since different proteins with similar molecular weights may migrate differently due to their differences in secondary, tertiary or quaternary structure. SDS is an anionic detergent, which is taken to unfold the proteins from their primary structures, and binds to the polypeptide backbone in a ratio of ~ 1.4 g SDS per gram of protein. The bound SDS gives the proteins a net negative charge per unit mass, overwhelming the intrinsic electrical charge of the sample protein. Additionally, SDS disrupts secondary and tertiary structures. Altering amounts of acrylamide and cross-linker controls the pore size, and thus the sieving properties of the polyacrylamide gel. %T is the total weight of acrylamide and cross-linker expressed as the percentage of the total volume and %C is the weight of the cross-linker expressed as the percentage of the total weight of both the acrylamide and cross-liner. Homogeneous gels (with same %T and %C) offer best resolution while gradient gels (varying %T and constant %C) provide a wider linear separation of molecular weight, minimized diffusion and sharp protein spots. The most common buffer system uses glycine as the trailing ion as described by Laemmli (Laemmli, 1970).

In our research, 12% SDS-PAGE (40% acrylamide mix; 375 mM Tris-HCl, pH 8.8; 0.1 % SDS; 0.1 % APS; 0.04 % TEMED), 0.75 mm thick gels were prepared. After polymerization, 25 µg of each protein sample was mixed with sample buffer upon the ratio of 4:1 and the mixture was heated at 65 ºC for 5 min, then the mixture was loaded per lane in the gel and kept one lane was available for molecular-weight standards. The gel was run at a constant voltage of 150 V for about 4 – 5 h. Then the gel was removed carefully from the glass for the further western blotting analysis.

3.5.3 Western Blotting

Western blotting is an analytical method that involves the immobilization of proteins on membranes before detection using monoclonal or polyclonal antibodies. The method was first described by Towbin (Towbin, Staehelin et al., 1979), but the official name of western blot was announced until 1981 by Burnette (Burnette, 1981).

When the protein electrophoresis was finished, the proteins were transferred onto a PVDF membrane following the "sandwich" protocol. All layers were mounted on a transfer chamber, and submerged in transfer buffer. The transfer process was carried out at 48 V for 4 hours. The PVDF membrane was removed from the chamber and incubated in blocking buffer overnight at 4 ºC.

3.5.4 Immunological detection of target proteins

The PVDF membrane was incubated with different primary antibodies at room temperature for 60 min. Then the membrane was washed three times in TBS-T buffer and then put into blocking buffer containing secondary antibody for 60 min. After all these steps, the PVDF membrane was washed for 30 min in TBS-T. Enhanced chemiluminiscence (ECL) detection was performed according to the manufacturer's instructions. ECL is a common technique for a variety of detection assays in biology. This technique is based on the oxidation of a luminol-based substrate by horseradish peroxidase. The horseradish peroxidase is conjugated to the primary antibody or to the secondary antibody. Light emission can easily be captured and visualized by exposing the blot to film or the charge coupled device (CCD) camera of an imaging system (Wang and Ren, 2005). The density of each protein band was analyzed by using ImagineJ software.

Table 7. Antibodies used in western blotting

Antibodies used in Western Blot		
Primary antibodies		
Targets	**Origin**	**Supplier**
actin C_4	Mouse monoclonal IgG1	MP Biomedicals
γH2AX-aa134-142 human	Mouse monoclonal IgG1	UPSTATE
p53 DO-1-aa11-25 human	Mouse monoclonal IgG2a	Santa Cruz
p21-aa145-164 human	Mouse monoclonal IgG1	BD Biosciences
c-Myc, p67	Mouse monoclonal	Santa Cruz
Secondary antibodies		
Targets	**Conjugated**	**Supplier**
Anti-mouse	Horseradish peroxidase	dianova, mouse 115-035-062

3.6 SA-β-gal ANALYSIS

Senescence associated β-gal (SA- β-gal) staining technology was initially proposed by Dimri (Dimri, Lee et al., 1995), and became nowadays widely used method to detect senescent cells.

Lysosomal β-galactosidase activity can be detected in situ in most mammalian cells by means of a cytochemical assay normally carried out at pH 4 (Dimri, Lee et al., 1995; van der Loo, Fenton et al., 1998), and β-galactosidase activity displays specifically at pH 6 in senescent cells, but not in quiescent or terminally differentiated cells (Dimri, Lee et al., 1995). A production of blue-dyed precipitate is conducted in senescent cell by the cleavage of the chromogenic substrate X-Gal.

3.6.1 Cell fixation

Cell samples were prepared in 6-well plates. After the treatment, control and treated cells were washed in PBS. Cells were fixed for 3 min at room temperature in 1 ml of fixative solution (See Buffer and Solution). Cells were washed again in PBS after the fixation and 2 ml of SA-β-gal staining solution was added into each well. The cells were incubated at 37 °C (CO_2 free) for 8 - 16 hours depending on the cell type.

3.6.2 β-gal activity detection by microscopy

The plate was taken out of the incubator when the blue dye could be observed under the microscopy. The SA-β-gal staining solution was discarded, refilled up each well with proper volume of water. The blue dye was then observed and photographed under microscope with different magnificence.

4 RESULTS

4.1 CYTOTOXICITY OF HARMINE, EMETINE AND SANGUINARINE AGAINST HUMAN CANCER CELLS

One of most outstanding characters of alkaloids is cellular cytotoxicity towards animals and humans. The cytotoxic effect can be generated when cell membranes become leaky or when elements of cytoskeleton are inhibited (Wink, 1993; Wink, 2007), and it occurs also as a result of the molecular interaction of an alkaloid with one or several important targets present in a cell (Fig.1)(Wink, 2007).

In order to evaluate the cellular cytotoxic effects of harmine (1), emetine (2), and sanguinarine (3), several human cancer cells including MCF-7, HeLa, and SiHa were used.
The cellular viability in control and in different human malignant cell lines in vitro was assessed with a microassay with tetrazolium salt (MTT) for quantitation of cells. The IC_{50} values after 24 h exposure were calculated after 24 h exposure by using the software of IC_{50} calculator (Microsoft Excel) (Fig 9a-i), and the data is presented in Table 8. In three independent assays the reduction of cancer cell viability was consistent. To compare the toxic potenties of the alkaloids, we applied further studied by using more specific concentration and prolonged treatment period in the same cells. Results showed that the alkaloids harmine, emetine, and sanguinarine exhibited a similar toxic effect in tested cells in a dose- and time-dependent manner.

The growth kinetics of all compound-treated cells initially differed from those of untreated or solvent treated control cells, regardless of the cell line used. The increasing concentrations of the compounds corresponded with the percentage of growth inhibition of all tested cells after different days of drug exposure. Harmine exhibited in vitro growth inhibitory activity against different cancer cell lines as determined in the MTT assay. During the exposure of harmine, the treated cells slowed their growth and showed an almost complete inhibition of proliferation after 24 h at the highest concentrations in all cells, the reduction of cell viability tested under highest concentration decreased to 37% in MCF-7 cell, 34% in HeLa cell, and 33% in SiHa cell of untreated control (Fig. 10a-c). Fig. 9d-f shows the effect of emetine on cell viability of MCF-7, HeLa and SiHa cells after 72 h treatment. Percent of cell viability was clearly affected by emetine in a dose- and time-dependent manner. Compared with the

dose-effect of emetine on MCF-7 cell, HeLa and SiHa cells revealed less sensitivity presented in platforms in response to emetine treatment (Fig. 10e,f). Emetine reduced the cell viability to 16% in MCF-7 cell, 13% in HeLa cell, and 14% in SiHa cell after 72 h treatment under the highest concentration applied in each cell line. As shown by the MTT reduction assay, treatment of cancer cells with various concentrations of sanguinarine for 72 h resulted in a dose-dependent decrease in the cell viability, while the cell viability decreased to 8.5% in MCF-7 cell, 25% in HeLa cell, and 2.5% in SiHa cell, respectively. All rates were calculated under the highest concentration of sanguinarine applied in each cell line (Fig. 10g-i).

Our results showed that alkaloid compounds 1-3 treatment had different effects on growth kinetics for the entire time of treatment in either cell line. The concentrations which induced an reduction of cell viability approximately around 30% and 50% were selected and summarized in Table 9.

(I) Cytotoxicity of harmine, emetine, and sanginarine in human cancer cells

(i) Cytotoxicity of **harmine** in cancer cells (24 h of exposure)

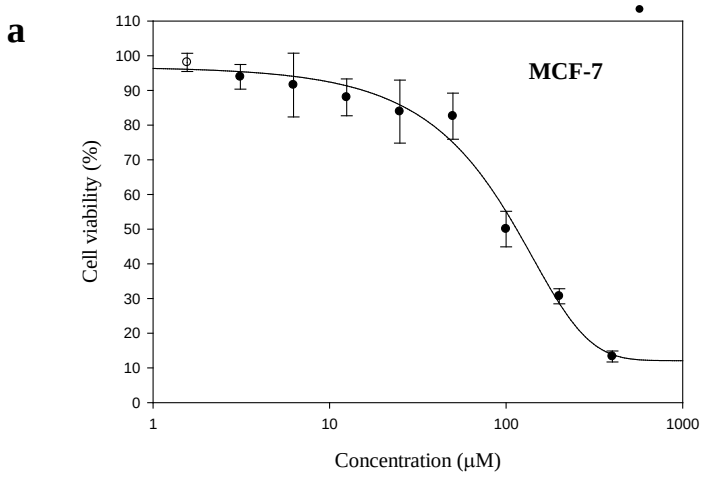


Fig 9a. The cytotoxicity of harmine in MCF-7 cells (error bars represent ± SD).

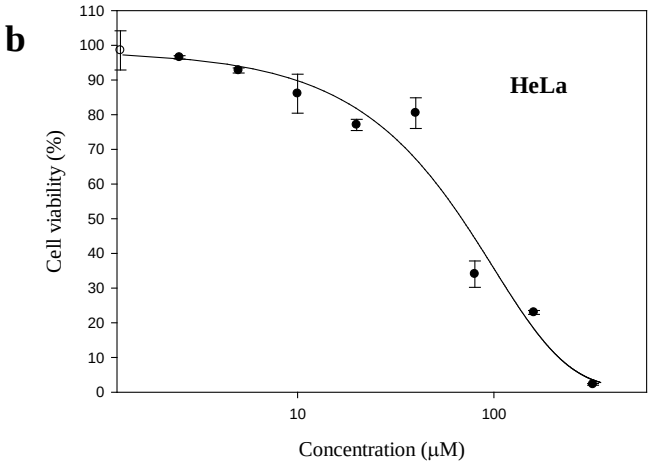


Fig 9b. The cytotoxicity of harmine in HeLa cells (error bars represent ± SD).

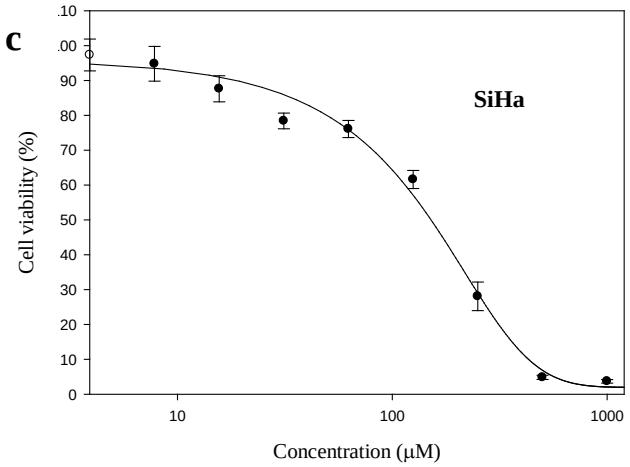


Fig 9c. The cytotoxicity of harmine in SiHa cells (error bars represent ± SD).

(ii) Cytotoxicity of **emetine** in human cancer cells (24 h of exposure)

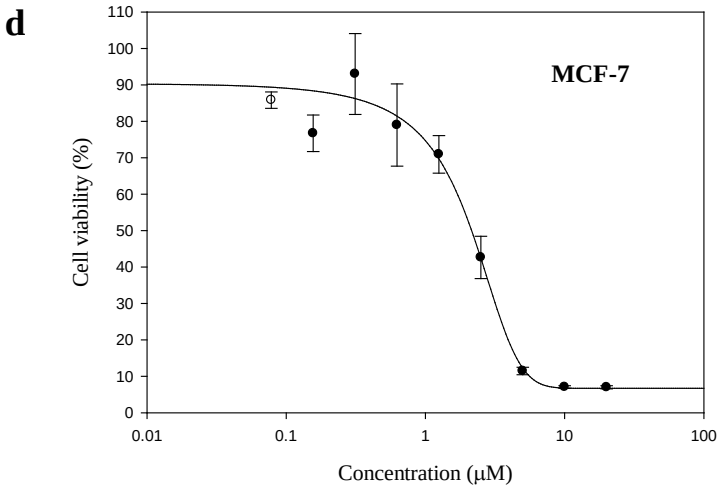


Fig 9d. The cytotoxicity of emetine in MCF-7 cells (error bars represent ± SD).

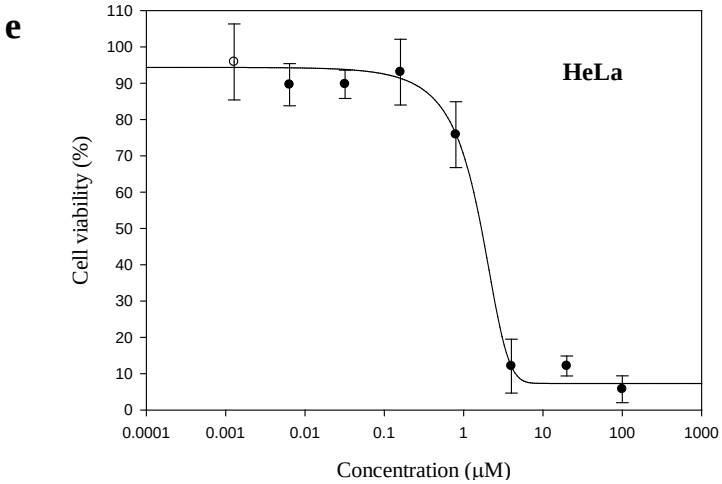


Fig 9e. Cytotoxicity of emetine in HeLa cells (error bars represent ± SD).

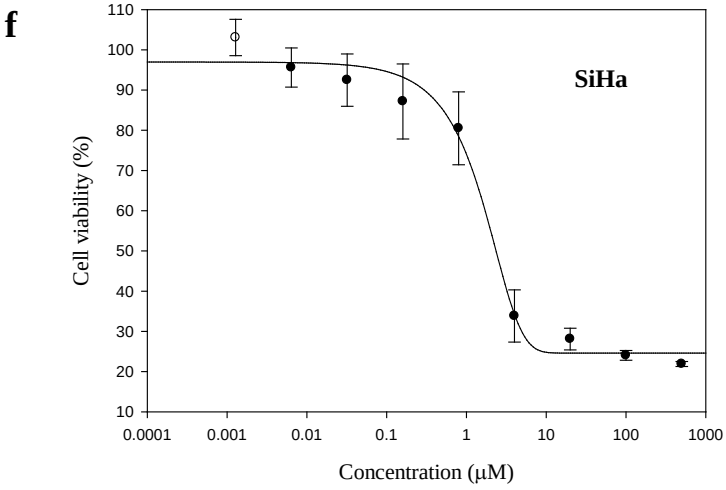


Fig 9f. Cytotoxicity of emetine in SiHa cells (error bars represent ± SD).

(iii) Cytotoxicity of **sanguinarine** in human cancer cells (24 h of exposure)

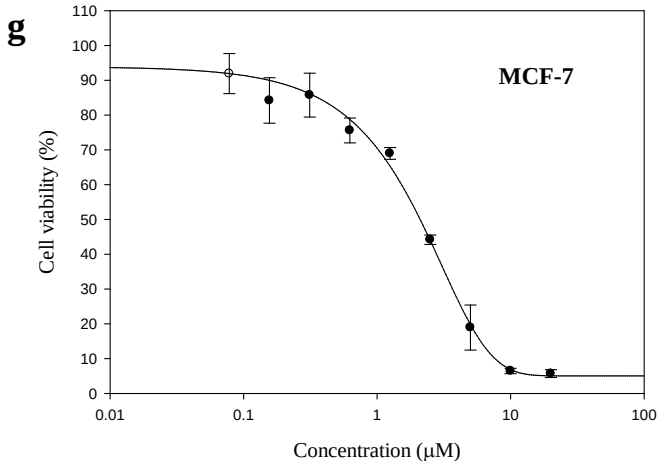


Fig 9g. Cytotoxicity of sanguinarine in MCF-7 cells (error bars represent ± SD).

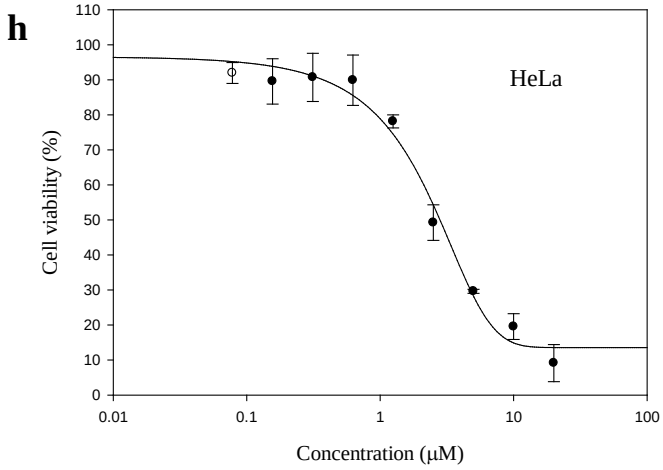


Fig 9h. Cytotoxicity of sanguinarine in HeLa cells (error bars represent ± SD).

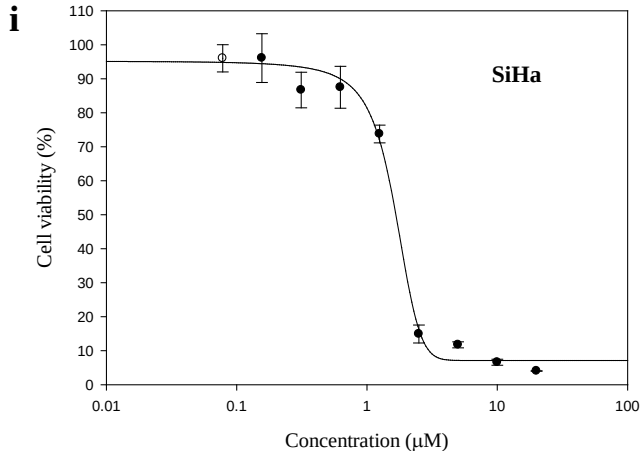


Fig 9i. Cytotoxicity of sanguinarine in SiHa cells (error bars represent ± SD).

(II). Anti-proliferative effect of alkaloids on cancer cells

(i) Anti-proliferative effect of **harmine** on human cancer cells

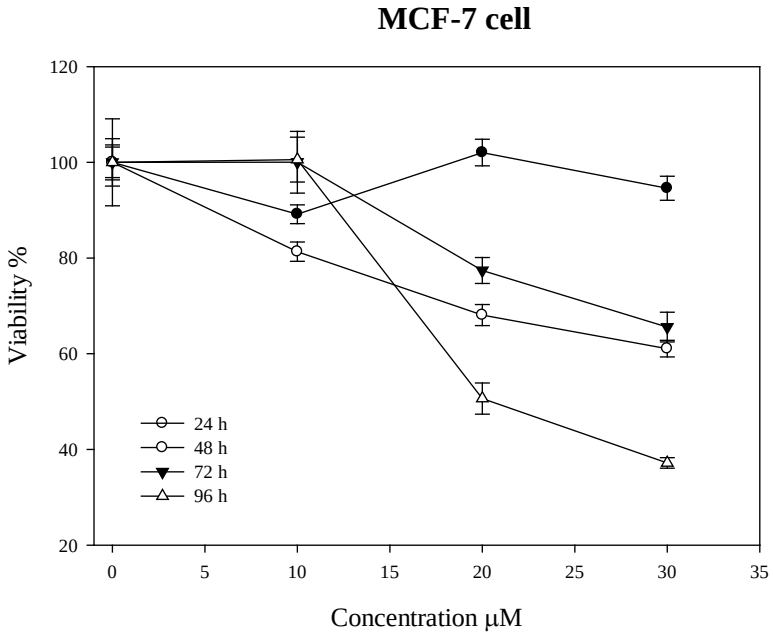


Fig 10a. The inhibitory effect of harmine on MCF-7 cell proliferation. Cells were exposed to various concentrations of harmine (10 µM, 20 µM, 30 µM), DMSO treated control was included (error bars represent ± SD).

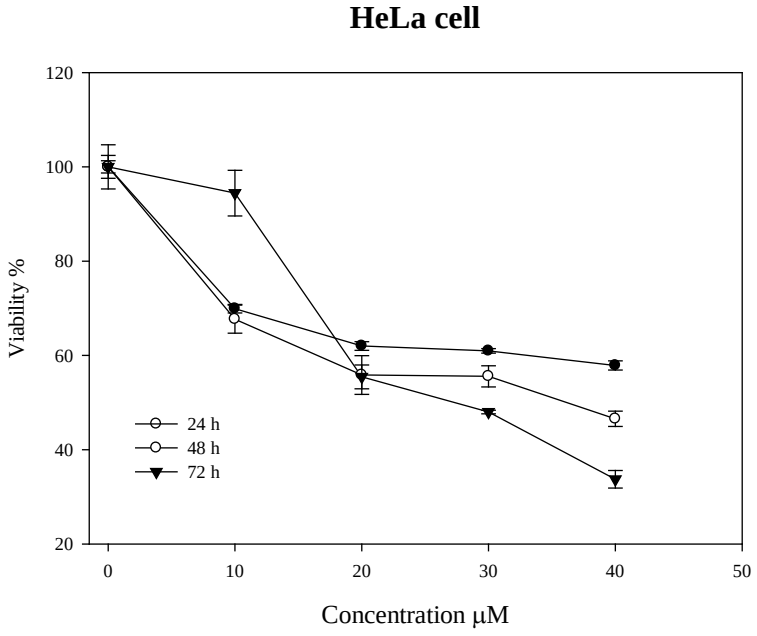


Fig 10b. The inhibitory effect of harmine on HeLa cell proliferation. Cells were exposed to various concentrations of harmine (10 µM, 20 µM, 30 µM, 40 µM). DMSO treated control was included (error bars represent ± SD).

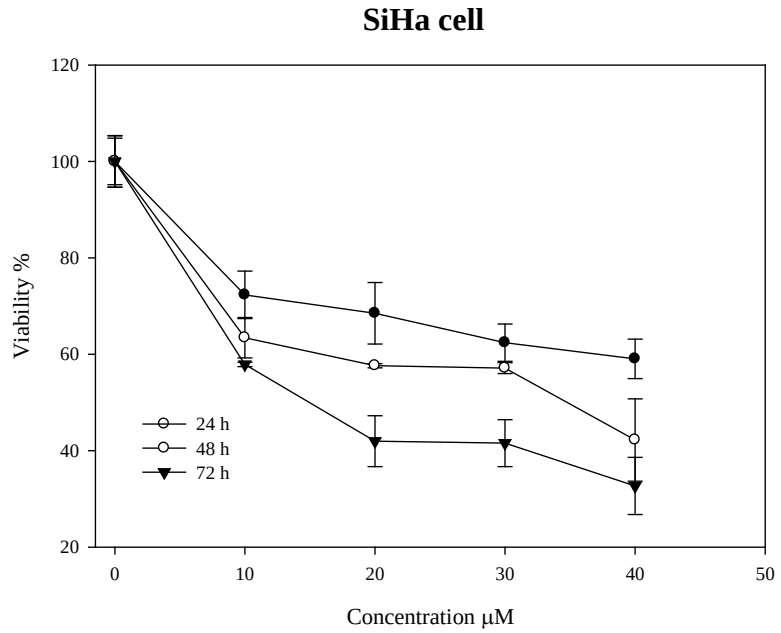


Fig 10c. The inhibitory effect of harmine on SiHa cell proliferation. Cells were exposed to various concentrations of harmine (10 µM, 20 µM, 30 µM, 40 µM). DMSO treated control was included (error bars represent ± SD).

(ii) Anti-proliferative effect of **emetine** on human cancer cells

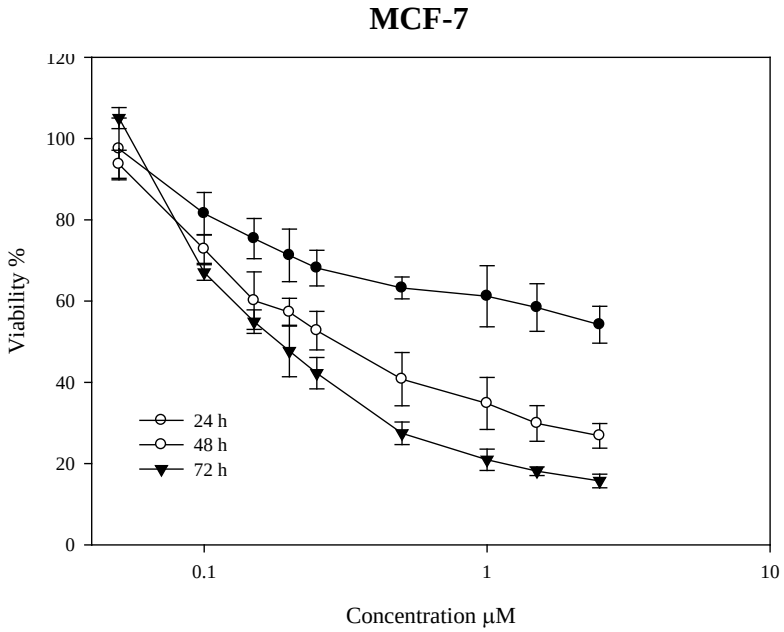


Fig 10d. **The inhibitory effect of emetine on MCF-7 cell proliferation.** Cells were exposed to various concentrations of emetine (0.05 µM, 0.1 µM, 0.15 µM, 0.2 µM, 0.25 µM, 0.5 µM, 1 µM, 1.5 µM and 2.5 µM) (error bars represent ± SD).

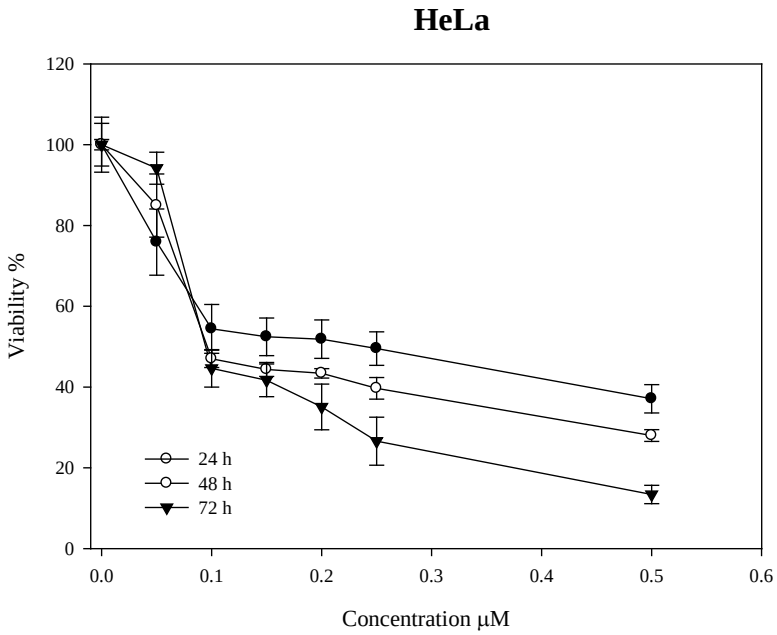


Fig 10e. **The inhibitory effect of emetine on HeLa cell proliferation.** Cells were exposed to various concentrations of emetine (0.05 µM, 0.1 µM, 0.15 µM, 0.2 µM, 0.25 µM and 0.5 µM) (error bars represent ± SD).

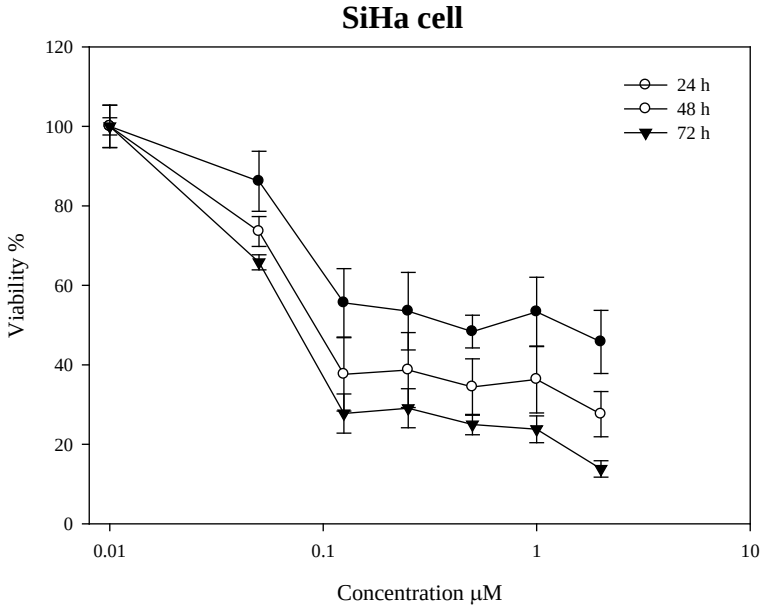


Fig 9f. **The inhibitory effect of emetine on SiHa cell proliferation.** Cells were incubated with various concentrations of emetine (0.05 µM, 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, 2 µM) (error bars represent ± SD).

(iii) Anti-proliferative effect of **sanguinarine** on human cancer cells

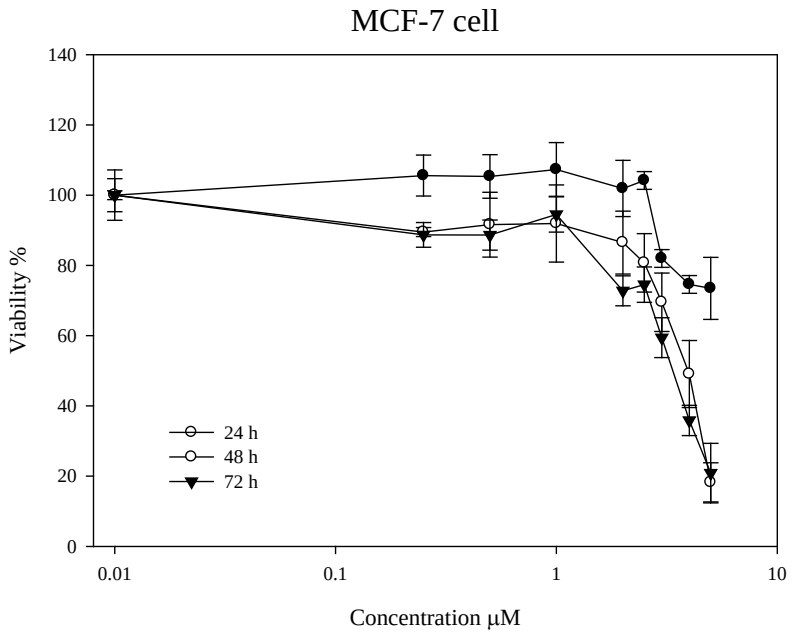


Fig 10g. **The inhibitory effect of sanguinarine on MCF-7 cell proliferation.** Cells were incubated with various concentrations of sanguinarine (0.25 μM, 0.1 μM, 1 μM, 2 μM, 2.5 μM, 3 μM, 4 μM and 5 μM) (error bars represent ± SD).

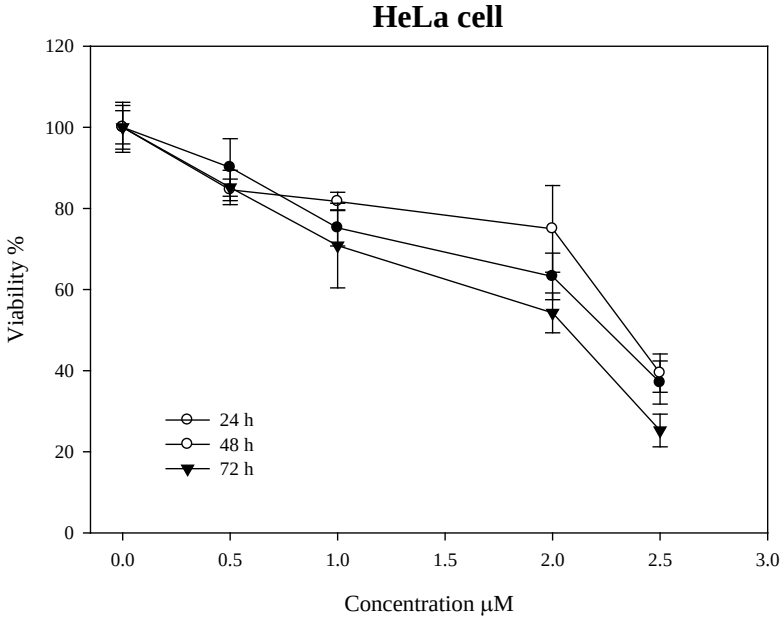


Fig 10h. **The inhibitory effect of sanguinarine on HeLa cell proliferation.** Cells were incubated with various concentrations of sanguinarine (0.5 μM, 1 μM, 2 μM and 2.5 μM) (error bars represent ± SD).

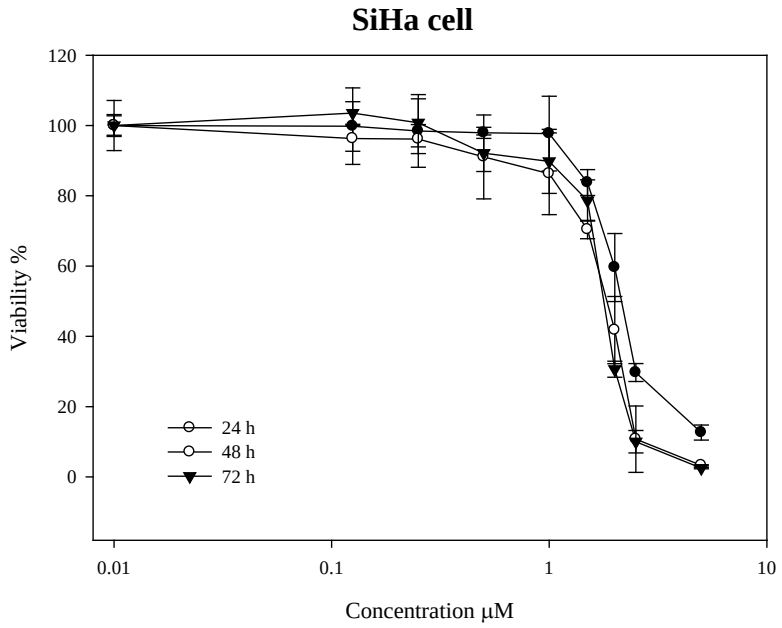


Fig 10i. **The inhibitory effect of sanguinarine on SiHa cell proliferation.** Cells were incubated with various concentrations of sanguinarine (0.1 μM, 0.25 μM, 0.5 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM and 5 μM) (error bars represent ± SD).

Table 8. The IC_{50} values of alkaloids in human cancer cells (24 h of exposure)

Compound	**IC_{50} (μM) / Cell line**		
	MCF-7	HeLa	SiHa
harmine	104.03 ± 6.71	73.37 ± 3.45	151.68 ± 8.44
emetine	1.96 ± 0.21	2.08 ± 0.26	2.77 ± 0.54
sanguinarine	2.22 ± 0.05	2.12 ± 0.30	1.66 ± 0.04

Table 9. The effect of alkaloids1-3 on HeLa cell proliferation

Cell line	**Compound**	**Inhibition efficiency**	
		Concentration (μM)	**cell growth inhibition (%)**
HeLa	1, Harmine	30	28.24 ± 4.44 (48 h)
	2, Emetine	0.15	50.93 ± 7.69 (48 h)
	3, Sanguinarine	2	25.09 ± 7.05 (48 h)

4.2 THE EFFECT OF ALKALOIDS ON TELOMERASE ACTIVITY

As we described earlier, usually anticancer drugs especially alkaloids are able to interact with several important targets which are present in a cell, such intercalation leads to a cell cycle arrest and/or cell death through influencing DNA replication, transcription or reverse transcriptase, etc (Wink, 2007). TRAP assay is widely used and a specific method for the evaluation of telomerase activity. Telomerase activity is then represented in PAGE gel as a ladder of bands differed by 6 base pair in length from each. In this study, the effect of harmine, emetine, and sanguinarine on cancer cell telomerase activity was investigated by using TRAP assay, and telomerase activity has been proved positively in MCF-7, HeLa, and SiHa cells (Okayasu *et al.*, 1998; Kurvinen *et al.*, 2006; Padmanabhan *et al.*, 2006).

4.2.1 The effect of harmine on human cancer cell telomerase activity

To examine the effect of harmine on telomerase activity, human cancer cells were cultured with it in different concentrations for various time periods. The investigated concentrations in TRAP assay were based on the proliferation assay. Samples were collected and analyzed simultaneously. The results showed that compared to untreated cells, harmine caused a significant inhibition of the telomerase activity in MCF-7 cells, and the maximal repression was detected at 20 μM for 96 h (Fig 11A). The telomerase inhibition percentage was calculated from band intensity, the expression was decreased by 81.87% of untreated control, and the inhibition occurred in a dose- and time-dependent manner (Fig 11B,C). The investigation was performed in both HeLa and SiHa cells as well by using the same method. Harmine inhibited telomerase activity in both cell lines, but a severe decrease of telomerase activity was only detected in HeLa cells, the inhibition revealed as early as 24 h at 30 μM and became almost undetectable at 48 h by 87% reduction of telomerase activity of untreated control (Fig 12C). Compared to HeLa cell, harmine initiated a moderate inhibitory effect on SiHa cell, a decrease of telomerase activity was detected to 46.55% at the highest concentration of 30 μM at 48 h (Fig. 13). Taken together, the results indicate that harmine induces a significant inhibition in human cancer cells, the reduction was in time- and dose-dependent manner in MCF-7 and HeLa cell lines (Fig 11B,C, Fig 12B,C).

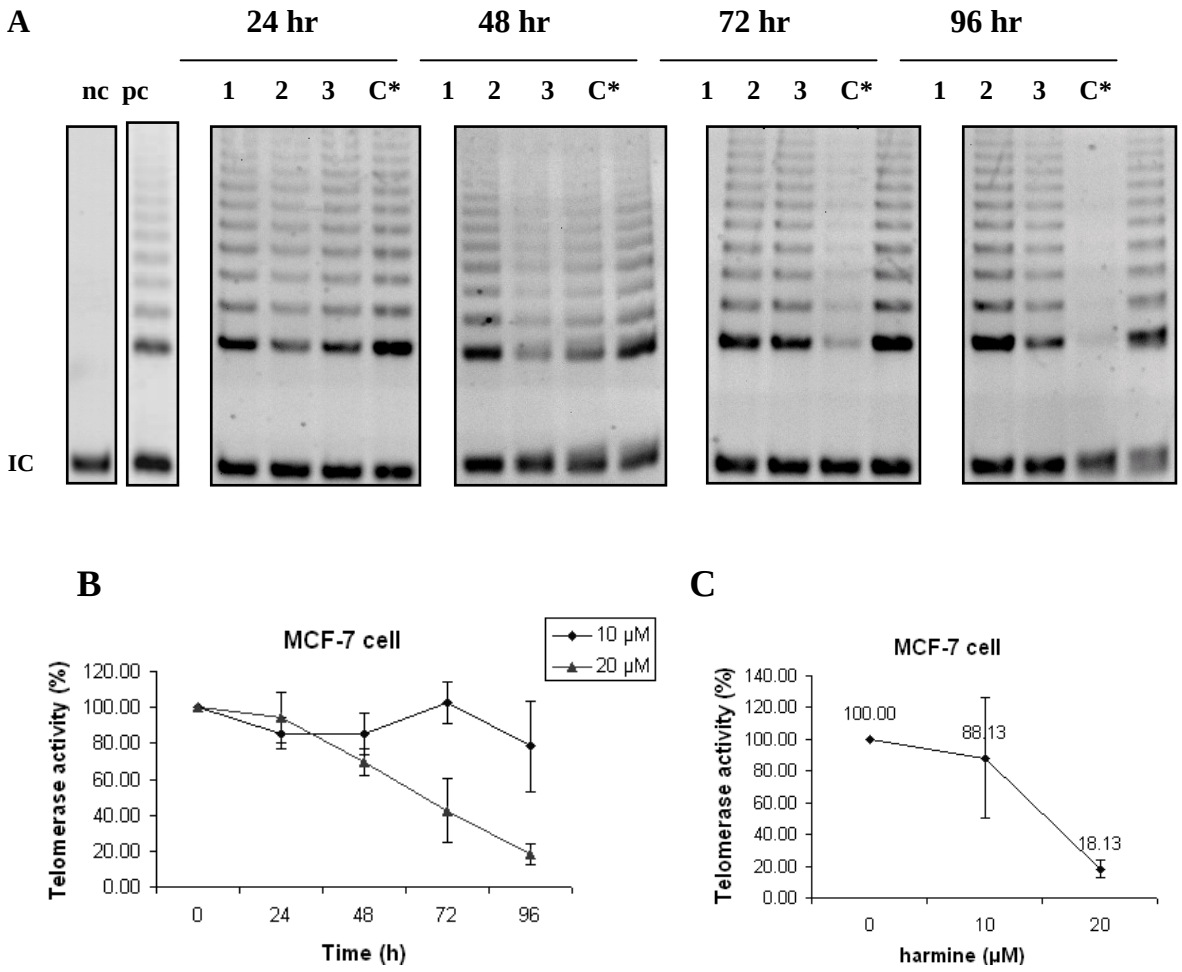


Fig 11. The effect of harmine on telomerase activiy in MCF-7 cells. (**A)**, nc: CHAPS buffer; pc: telomerase-positive control; C*, DMSO treated control; IC: internal control; lane 1, untreated control; lane 2-3, cells treated with 10, 20 μM of harmine, respectively; Graphical representation of telomerase activity from densitometric analysis in **(B)** time-dependent manner or **(C)** dose-dependent manner. Each point represents the mean value of telomerase activity ± S.D. from three independent experiments.

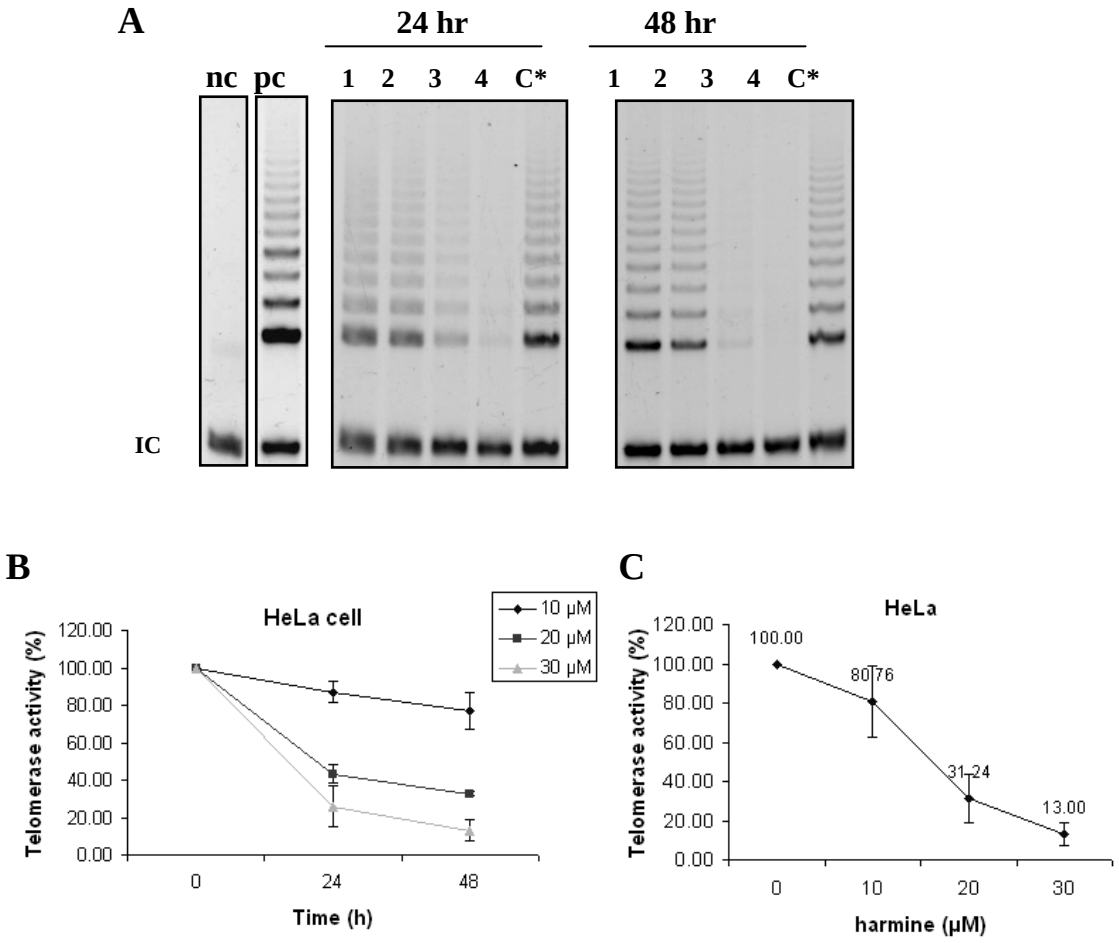


Fig 12. The effect of harmine on telomerase activiy in HeLa cells. (**A)**, nc: CHAPS buffer; pc: telomerase-positive control; C*, DMSO treated control; IC: internal control; lane 1, untreated cells; lane 2-4, cells were treated with 10, 20, 30 μM of harmine; Graphical representation of telomerase activity from densitometric analysis in **(B)** time-dependent manner or **(C)** dose-dependent manner. Each point represents the mean value of telomerase activity ± S.D. from three independent experiments.

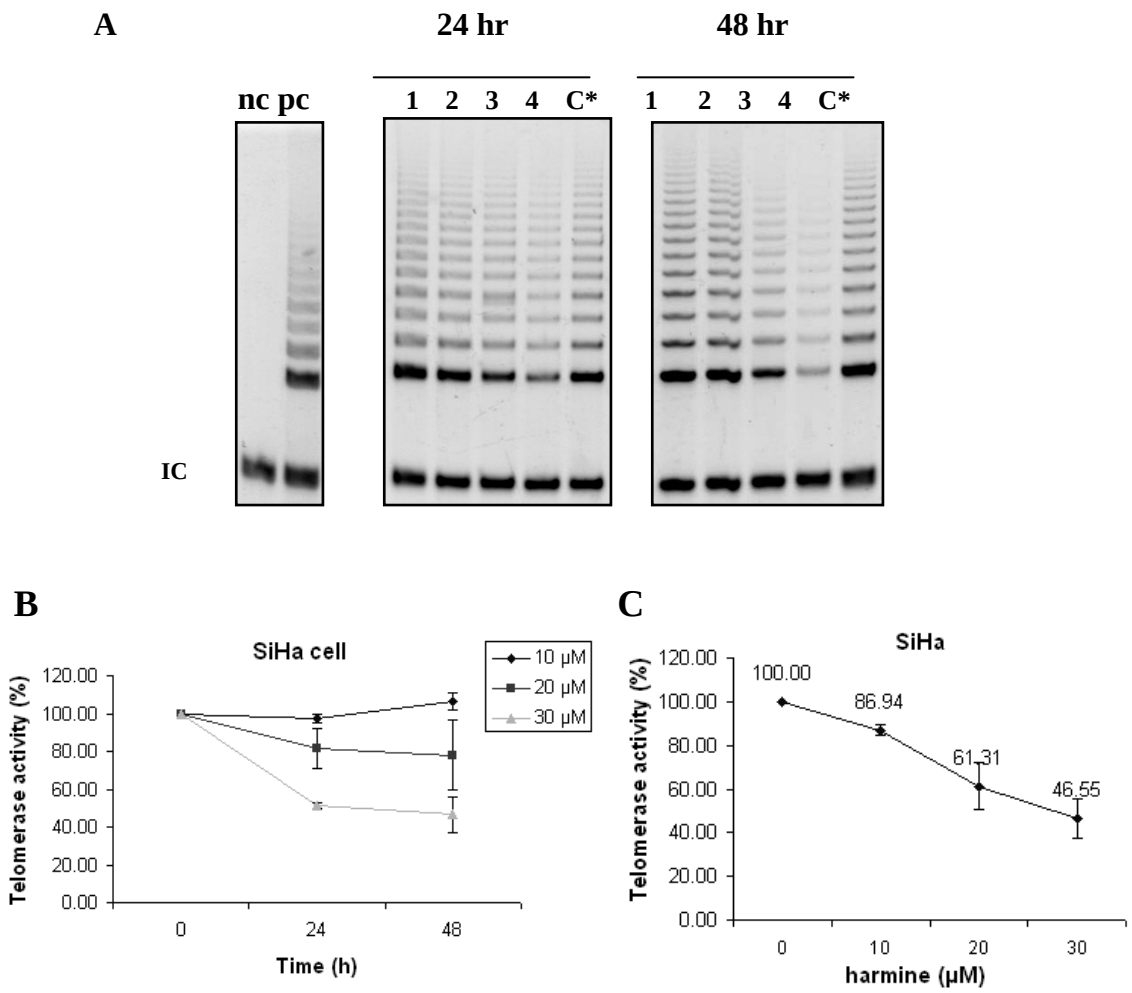


Fig 13. The effect of harmine on telomerase in SiHa cells. (A), nc: CHAPS buffer; pc: telomerase-positive control; C*, DMSO treated control; IC: internal control; lane 1, untreated cells; lane 2-4, cells were treated with 10, 20, 30 μM of harmine; Graphical representation of telomerase activity from densitometric analysis in **(B)** time-depedent manner or **(C)** dose-dependent manner. Each point represents the mean value of telomerase activity ± S.D. from three independent experiments.

4.2.2 The effect of emetine on telomerase activity in cancer cell

To examine the effect of emetine on human telomerase, the same technique and analysis method were used with the cellular extracts of HeLa and SiHa cells. As the result showed in Table 9, emetine at 0.15 μM or 0.25 μM induced a common proliferative reduction of 50% in both cell lines after 48 h. Therefore, for the further assessment of telomerase activity, those two concentrations were taken as the highest concentration to be tested in HeLa and SiHa cells, respectively. As predicted in Fig 14A, a slight decrease of telomerase activity was

observed after the exposure of emetine at 0.15 µM in HeLa cells, and the reduction was not changed by the longer treatment period. The reduction was then calculated based on the intensity of each band as 27.72% and 30.85% of untreated control at 24 h and 48 h, respectively. The highest concentration of 0.25 µM induced a significant reduction of telomerase activity of HeLa cell (Fig. 14B). The analysis of telomerase inhibition suggests that emetine inhibits HeLa cell telomerase in dose-dependent manner. Compared with the inhibitory effect on HeLa cells, emetine exhibited a weaker inhibitory effect on SiHa cell telomerase (Fig 15A), the expression level was decreased to 90.10% after 48 h at 0.25 µM and the reduction was increased to 65.01% at the highest concentration of 0.5 µM. The rate was not altered significantly by the extended incubation period.

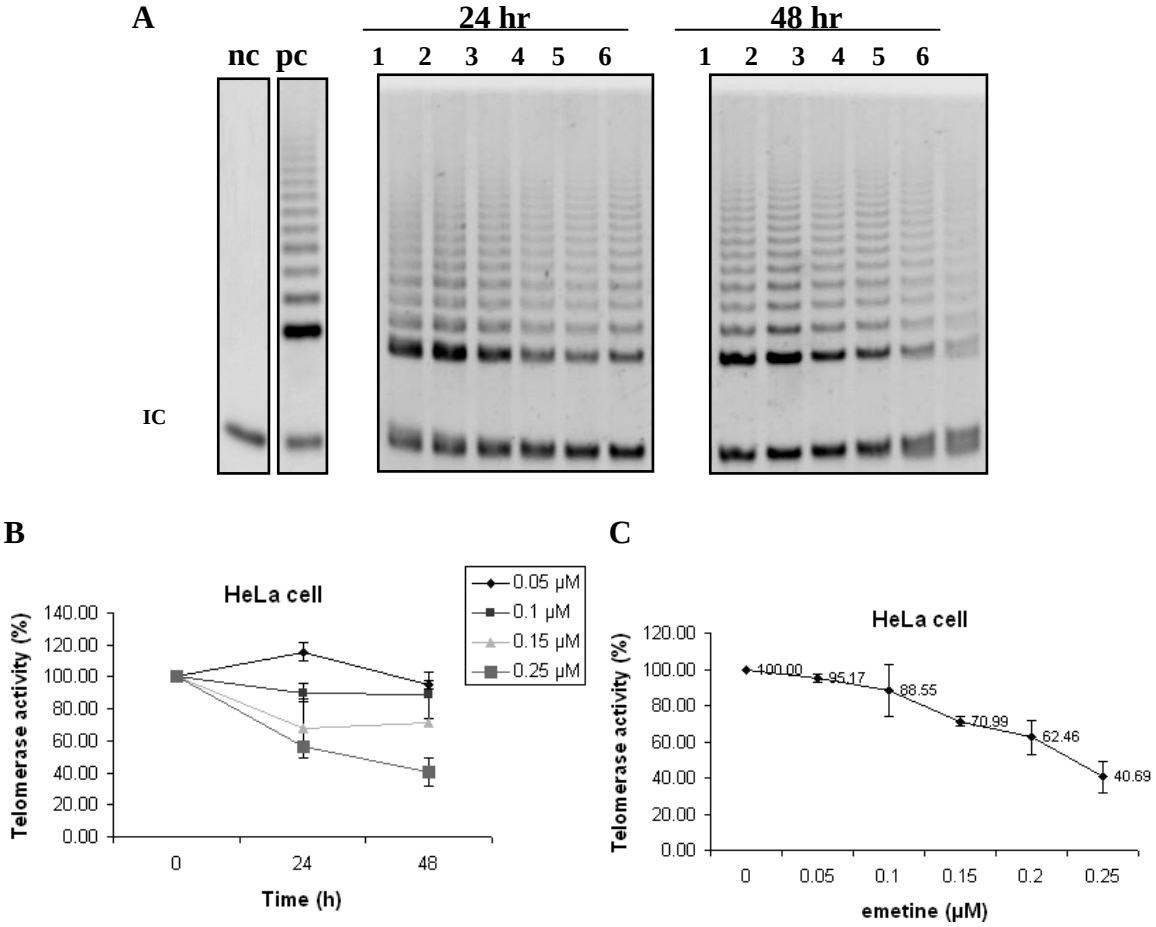


Fig 14. The effect of emetine on telomerase activity in HeLa cells. (A), nc: CHAPS buffer; pc: telomerase-positive control; IC: internal control; lane 1, untreated cells; lane 2-6 : cells were treated with emetine in turn in 0.05 µM, 0.1 µM, 0.15 µM, 0.2 µM, and 0.25 µM. Graphical representation of telomerase activity from densitometric analysis in **(B)** time-depedent manner or **(C)** dose-dependent manner. Each point represents the mean value of telomerase activity ± S.D. from three independent experiments.

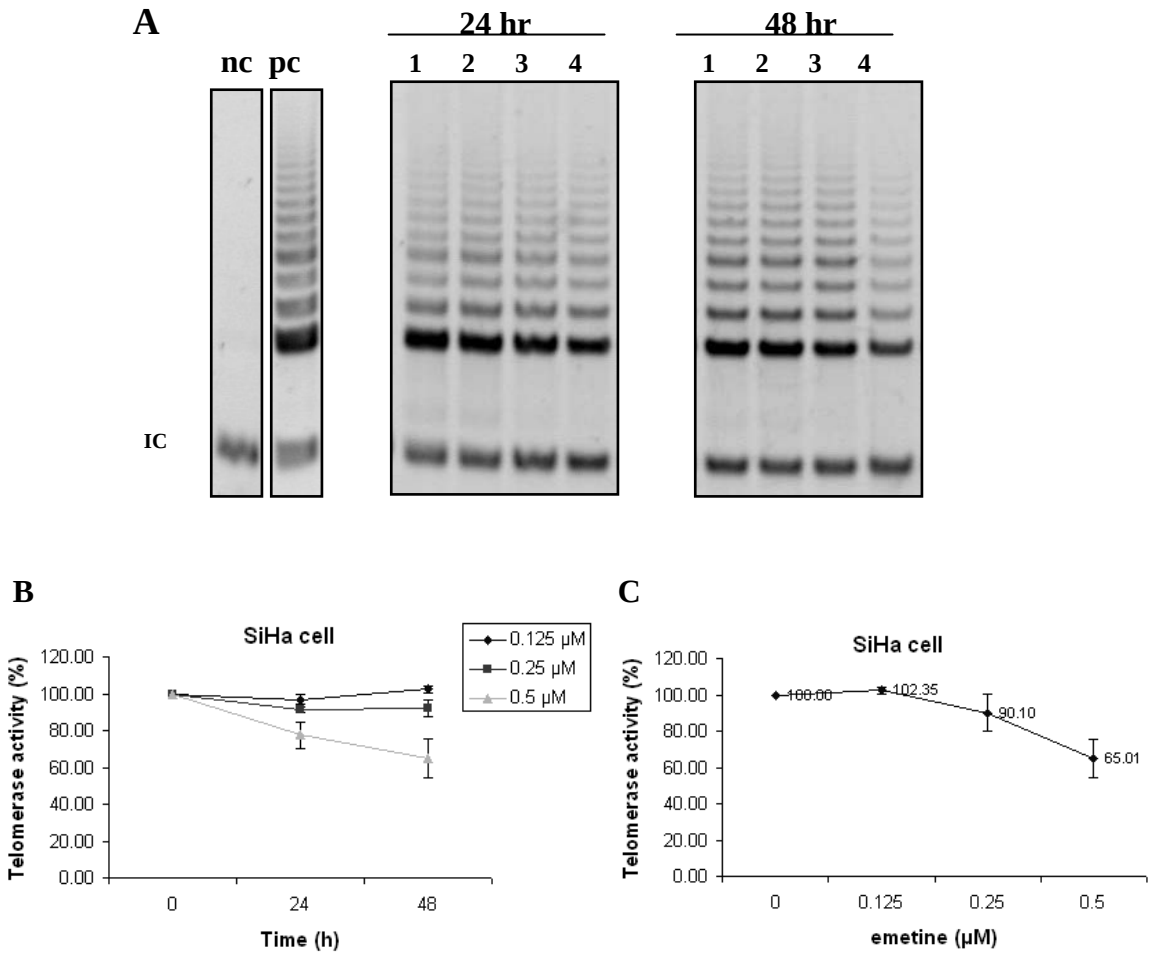


Fig 15. The effect of emetine on telomerase activity in SiHa cells. (A), nc: CHAPS buffer; pc: telomerase-positive control; IC: internal control; lane 1, untreated cells; lane 2, cells were treated with 0.125 μM of emetine; lane 3, cells were treated with 0.25 μM of emetine; lane 4, cell were treated with 0.5 μM of emetine. Graphical representation of telomerase activity from densitometric analysis in **(B)** time-depedent manner or **(C)** dose-dependent manner. Each point represents the mean value of telomerase activity ± S.D. from three independent experiments.

4.2.3 The effect of sanguinarine on HeLa cell telomerase

Sanguinarine concentrations used in experiments of TRAP assay were indicated (Fig. 16). As showed in Fig 16, telomerase activity of HeLa cells was inhibited after 48 h treatment (Fig 16A), the telomerase activity was decreased to 39.79% of untreated control at 2.5 μM at 48 h.

No reduction of telomerase activity was detected in lower concentrations (0.5 µM and 1 µM) of sanguinarine (Fig 16B,C).

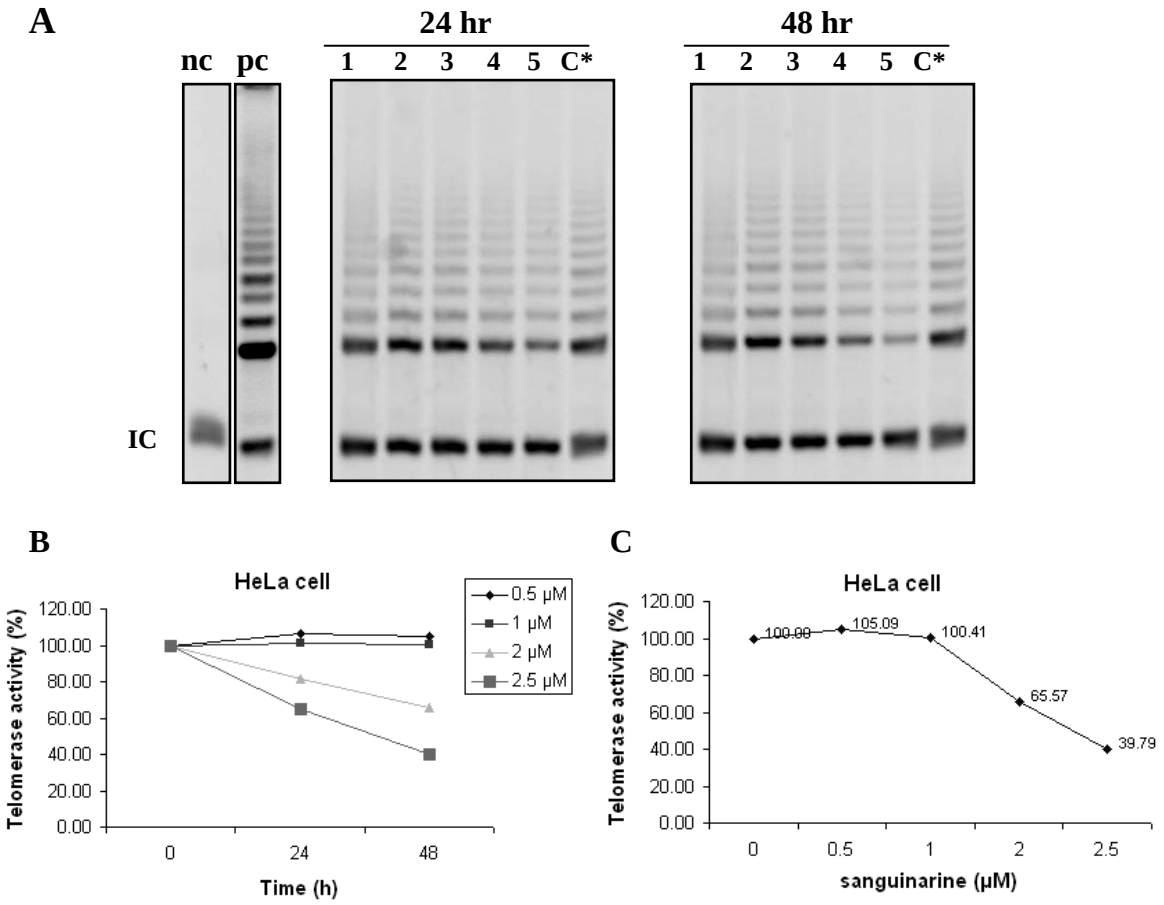


Fig 16. The effect of sanguinarine on HeLa telomerase activity. (A) nc: CHAPS buffer; pc: telomerase-positive control; C*, DMSO treated control; IC: internal control; lane 1, untreated cells; lane 2, cells were treated with 0.5 µM of sanguinarine; lane 3, cells were treated with 1 µM of sanguinarine; lane 4, cells were treated with 2 µM; lane 5, cells were treated with 2.5 µM of sanguinarine **(B)** Sanguinarine inhibits HeLa cell telomerase in time-dependent manner. **(C)** Sanguinarine inhibits HeLa cell telomerase in dose-dependent manner.

4.2.4 Harmine has no direct effect on the isolated telomerase protein

In order to elucidate the underlying mechanism of harmine-induced telomerase inhibition in MCF-7 and HeLa cells, a set of cell-free TRAP assay was carried out with the isolated extract from the cells. The cell-free system has been routinely used as a reference in evaluating the effects of standard chemotherapeutic agents on telomerase activity in human

cancer cell lines (Mosmann, 1983; Raymond, 1996; Zhu, 1996). In this experiment, solution of harmine at 30 and 40 µM was added to the telomerase reaction mixture containing 0.5 µg cell extract of each cell line, the mixture was incubated at room temperature for various time periods, and the assay was run. Fig 17 illustrates that no alternation of telomerase activity was found in response to harmine treatment compared to the untreated and solvent control (Fig 17). Telomerase known as a heat-sensitive protein, in this experiment, its activity was eventually diminished accompanied with the extended experimental temperature, and completely disappeared at 24 h in all tested cell extracts due to the enzyme lability. Due to these results we concluded that the inhibition of telomerase activity was not caused by the direct interaction between harmine and the enzyme.

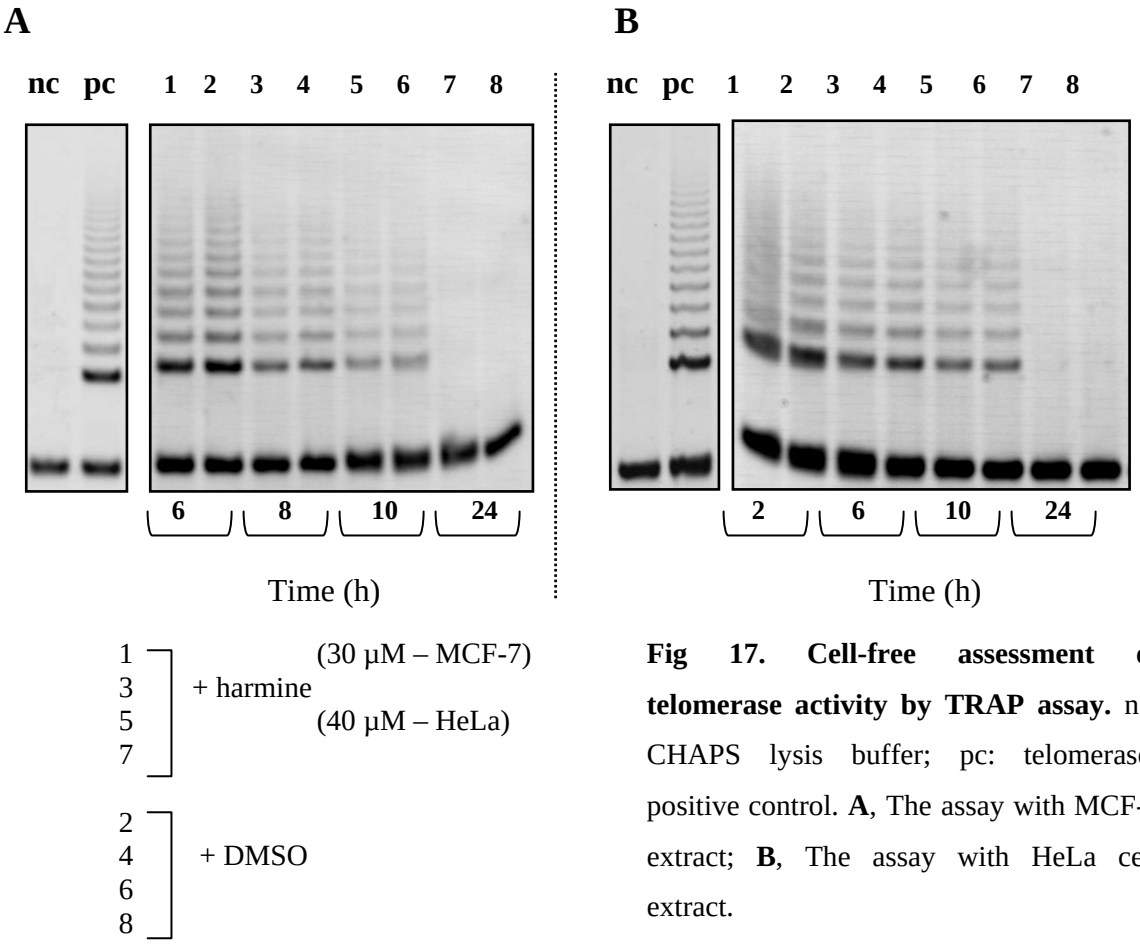


Fig 17. Cell-free assessment of telomerase activity by TRAP assay. nc: CHAPS lysis buffer; pc: telomerase-positive control. **A**, The assay with MCF-7 extract; **B**, The assay with HeLa cell extract.

4.2.5 Harmine inhibits MCF-7 and HeLa cell telomerase through different mechanisms

4.2.5.1 Harmine down-regulates the mRNA expression of hTERT

hTERT is a critical determinant of telomerase for telomerase catalytic activity (Kanaya *et al.*, 1998; Snijders *et al.*, 2004). The RNA template (hTR), as reported from different groups, is detectable in most of cells, including normal cells. However, several in vitro studies showed that hTR expression level was increased in response to the cellular stress (Yi *et al.*, 1999). Our previous investigation showed that harmine inhibits MCF-7 and HeLa cell telomerase activity via other mechanisms than the enzyme directly. Therefore, we next focused on the transcriptional regulation study on harmine-treated cells by using RT-PCR and the relative quantification PCR (Real-time PCR) techniques.

The investigation was split into two steps: in the first step, MCF-7 cells were treated with harmine in different concentrations (10, 20 µM) up to 48 h. In order to ensure equal amount of mRNA was reverse transcripted in all samples, β-actin control was used. PCR result showed that treatment of harmine at 10 µM did not alter the hTERT expression after 48 h, whereas a decrease was detected at 20 µM (Fig 18A). This result was later confirmed by the relative quantification PCR (Fig 19). Based on these results, the second step of analysis was performed. The cells were treated with the same concentration of harmine at 20 µM up to the various time periods (48 h, 96 h). A reduction of the hTERT expression was observed which stayed stable throughout the entire treatment time up to 96 h (Fig 18B), this result was also determined by the quantification PCR (Fig 20).

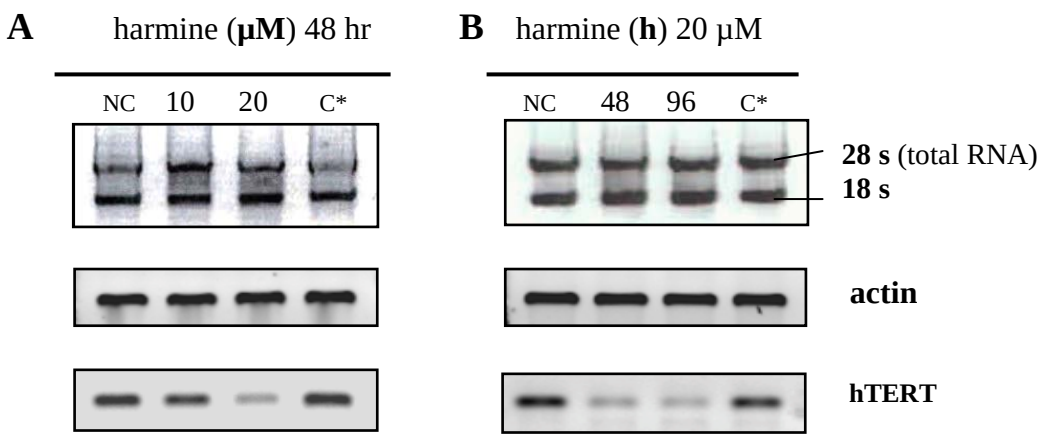


Fig 18. The overall expression of hTERT in response to harmine in MCF-7 cells. NC: untreated control; C*: DMSO control. **A**, The dose-effect study on the global expression of hTERT . **B**, The time-effect study on the global expression of hTERT.

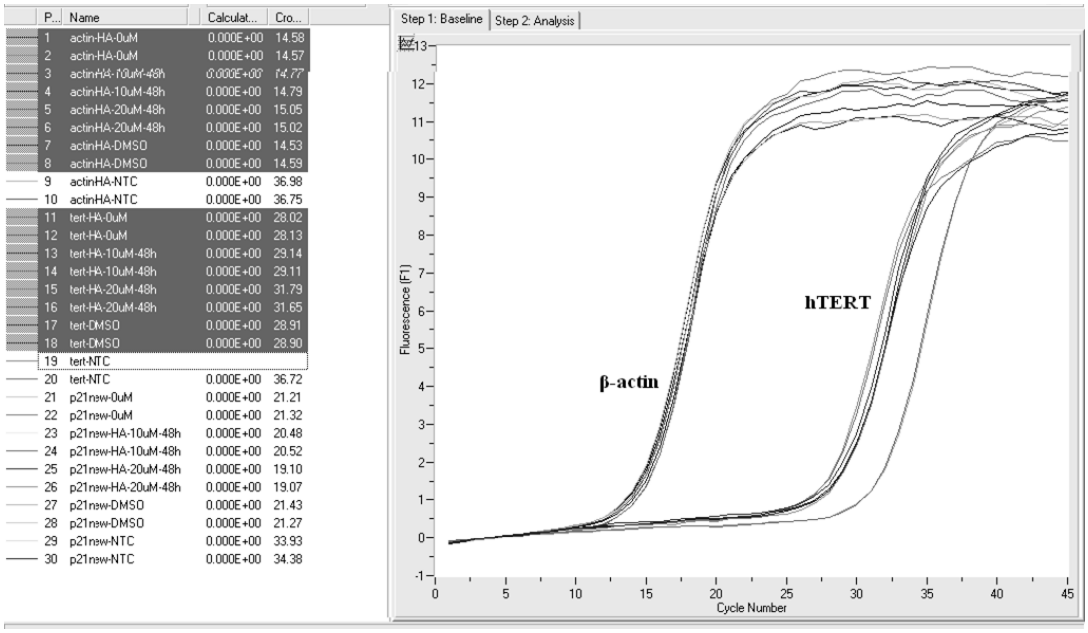


Fig 19. The relative quantification PCR anaylsis of hTERT expression in MCF-7 cells in dose-effect manner. The graph represents the fluorescence intensities versus cycle number where the PCR products were formed. Each curve representing an individual sample.

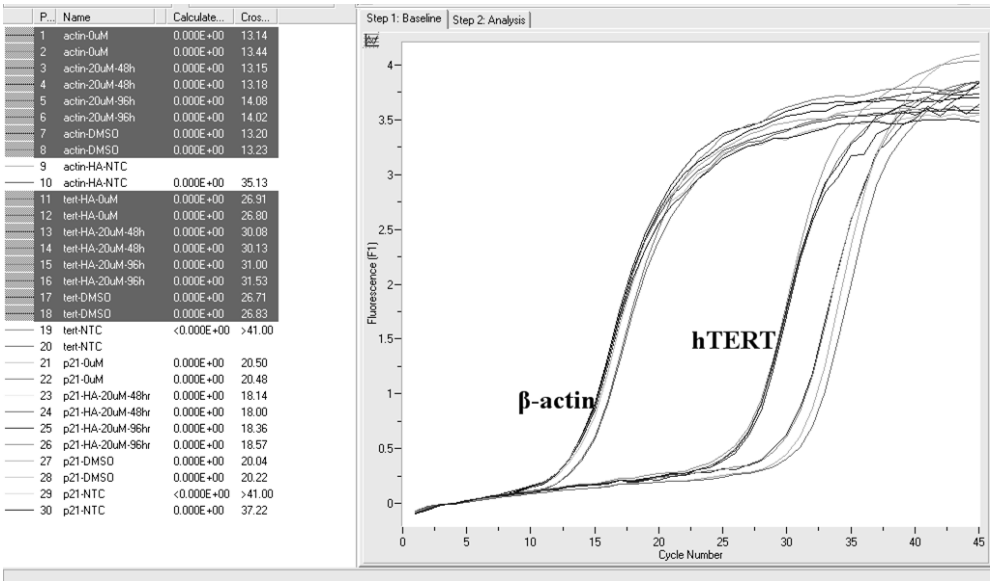


Fig 20. The relative quantification PCR anaylsis of hTERT expression in MCF-7 cells in time-effect manner. The graph represents the fluorescence intensities versus cycle number where the PCR products were formed. Each curve representing an individual sample.

The cp values were taken from at least three independent experiments, and analyzed by using the software of REST2008 (Munich, Germany). The graphical representation shows that harmine down-regulates MCF-7 cell hTERT expression (Fig 20).

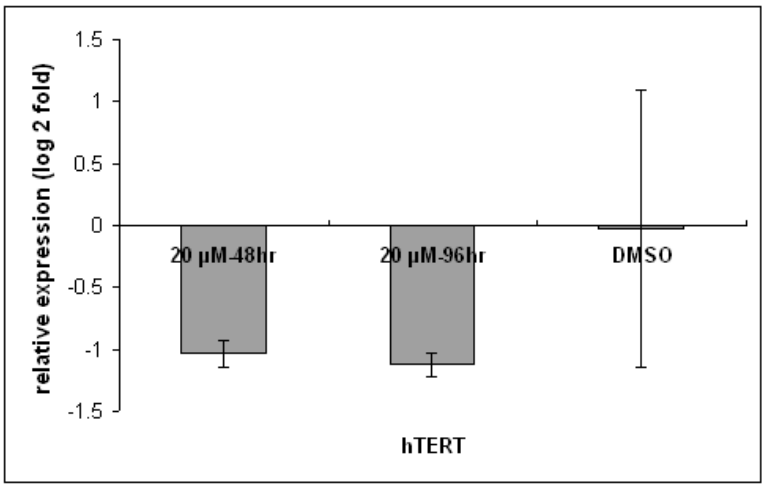


Fig 21. Real time PCR analysis of hTERT gene showing a down-regulation on treatment with harmine. Experiments were repeated three times independently. The column is represented as mean ± standard errors of the samples and is statistically significant ($p_{48\,h} = 0.003$, $p_{96\,h} = 0.002$).

4.2.5.2 Harmine altered all detectable transcripts of hTERT in MCF-7 cell

We have found that harmine markedly inhibited the global transcription of hTERT, we then asked whether the splicing of hTERT was involved in the regulation of telomerase activity in harmine-treated MCF-7 cells. hTERT transcript has at least six alternate splicing sites (4 insertion sites and 2 deletion sites) in different human cancer cells (Kilian *et al.*, 1997; Meyerson *et al.*, 1997), and all 4 insertions cause premature translation terminations. To examine expression of alternatively spliced hTERT with the full length functional and the defective RT region was examined using primers (hTERT_2026F, hTERT_2048R) spanning the RT region of hTERT (Fig 22A). This region contains potential α and β splicing sites. The hTERT mRNA with a full length transcript was identified as hTERT +α+β (fl). mRNAs containing a defective alternative splicing were identified as hTERT -α (α variant), hTERT -β (β variant), and hTERT -α-β (-α-β variant). Under our experimental conditions, the -α-β variant was permanently absent. Analysis of mRNA showed that all three detectable alternative splicing forms of hTERT transcripts were presented in untreated MCF-7 cells. In the dose-effect study, expression of the full-length variant mRNA decreased significantly in a dose-dependent manner after harmine treatment at 20 μM. Both α variant and β variant mRNA expression was also decreased (Fig 22B). In the time-effect study, all detectable variants including the full length, α variant and β variant mRNA were dramatically decreased upon treatment of harmine and became almost undetectable on at 96 h (Fig 22C). The variant's distribution assay demonstrated the pattern of hTERT splicing was altered. Harmine decreased the expression of both the functional and two non-functional hTERT mRNAs, suggesting that hTERT splicing might not play a role in harmine-induced down-regulation of telomerase activity in MCF-7 cells.

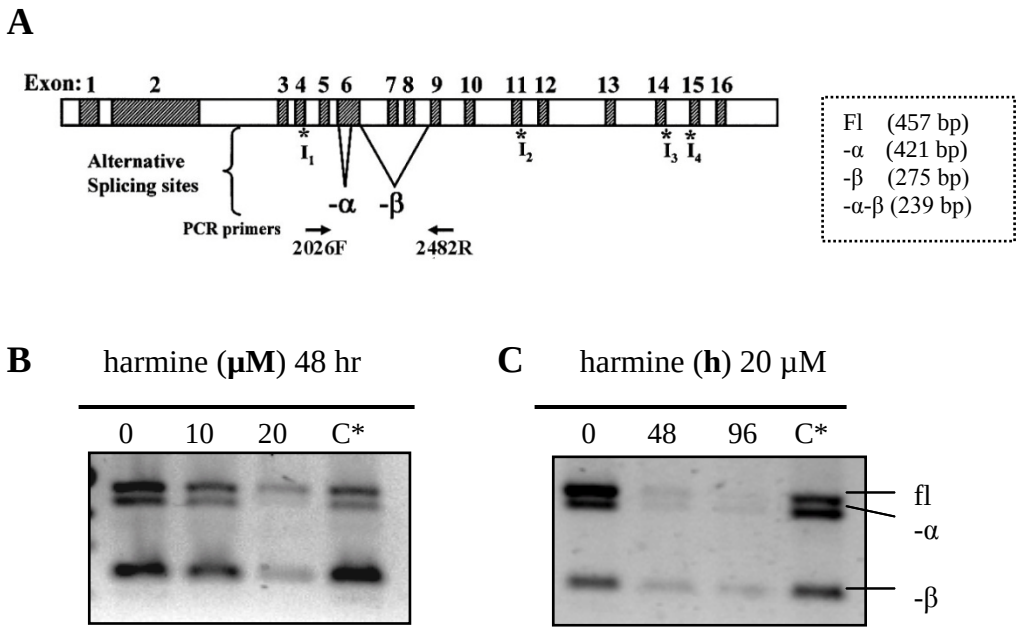


Fig 22. Alternative splicing variant expression of hTERT in response to harmine in MCF-7 cells. C*: DMSO control. **A**, Genomic organization of the hTERT gene. Exons 1–16 and alternative splicing insertions (I_1–I_4, indicated by asterisks) and deletions (-α and -β, indicated by solid lines) (Yi *et al.* 2001).**B**, The dose-effect study on the splicing pattern of hTERT expression.**C,** The time-effect study on the splicing pattern of hTERT expression.

4.2.5.3 Harmine induces hTERT alternative splicing variant shifting in HeLa cell

Since telomerase activity was affected dramatically by harmine in HeLa cells (Fig. 12A), a direct inhibition of the telomerase could be included. In order to find out the potential mechanism of telomerase regulation, a set of analysis was carried out by using the same techniques as in MCF-7 cells. However, data obtained from normal PCR and relative quantification PCR showed that no repression of the global transcription of hTERT was detected in HeLa cells (Fig 23a). We, therefore, asked whether modulation of the hTERT splicing pattern might be a potential mechanism of telomerase inhibition in the HeLa cells. Upon treatment of harmine, an alteration of the hTERT splicing was observed at 24 h, which led to a slight decrease of the full length transcript (+α+β) and an moderate increase of the β variant, whereas no alternation was found on the expression of α variant (Fig 23b). Further

prolongation of the treatment of 48 h did not increase the effect of harmine on hTERT splicing. Such alternation on the splicing pattern of hTERT transcript would probably account for the harmine-induced down-regulation of the telomerase enzymatic activity.

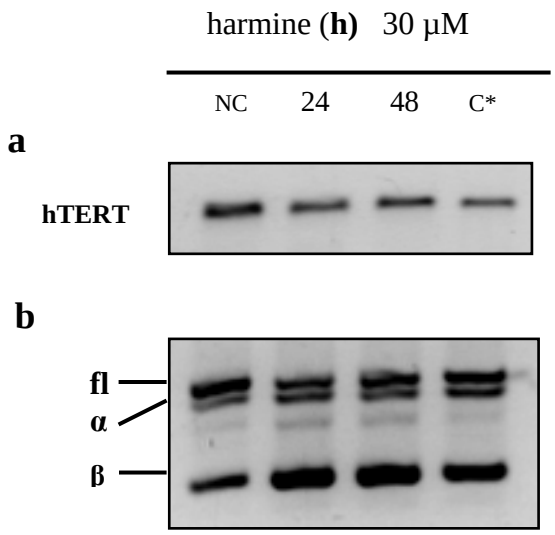


Fig 23. Alternative splicing variant expression of hTERT in response to harmine in HeLa cells. NC: untreated control; C*: DMSO control. **a**, The time-effect study on the global expression of hTERT. **b**, The time-effect study on the splicing pattern of hTERT expression.

Conclusion: harmine inhibits MCF-7 and HeLa cell telomerase activity through different mechanisms. Harmine induces a down-regulation on the hTERT mRNA expression in MCF-7 cells, whereas it initiates an alternation of hTERT splicing in HeLa cells.

4.3 HARMINE INDUCES ACCELERATED SENESCENCE IN MCF-7 CELL

Harmine triggers a mild DNA damage response in MCF-7 cell through p53/p21 pathway. As we know both apoptosis and senescence have been linked to p53 status (Elmore *et al.*, 2002; Offer *et al.*, 2002), but the determinant remains unknown. MCF-7 cells lack a functional form of caspase 3 (Janicke *et al.*, 1998), senescence might represent a default response in cells incapable of undergoing apoptosis. Even in other case that MCF-7 cells were manipulated to express caspase 3, cells also failed to undergo apoptosis (Janicke *et al.*, 2001). Therefore, we speculated that harmine might induce an accelerated senescence in MCF-7 cells. After harmine treatment, the cells were fixed and incubated with SA-β-gal buffer for about 10 hours until the blue dye could be observed under the microscope. Representative photomicrographs showing harmine-induced accelerated senescence of accelerated senescence induced by harmine exposure is presented in Figure 25. β-galactosidase

expression was present in the MCF-7 cells as early as 2 days (Fig 24b), and became intense and expressed in virtually every cell of the culture at 4 days (Fig 24d).Cells with positive dye were larger in size or multinucleated (indicated with arrows), both of which are morphological features indicative of a senescent state.The SA-β-gal staining was not detected or barely detected in untreated or solvent control cells. This result indicates that harmine induces MCF-7 cells enter accelerate senescence. The experiment was repeated at least twice.

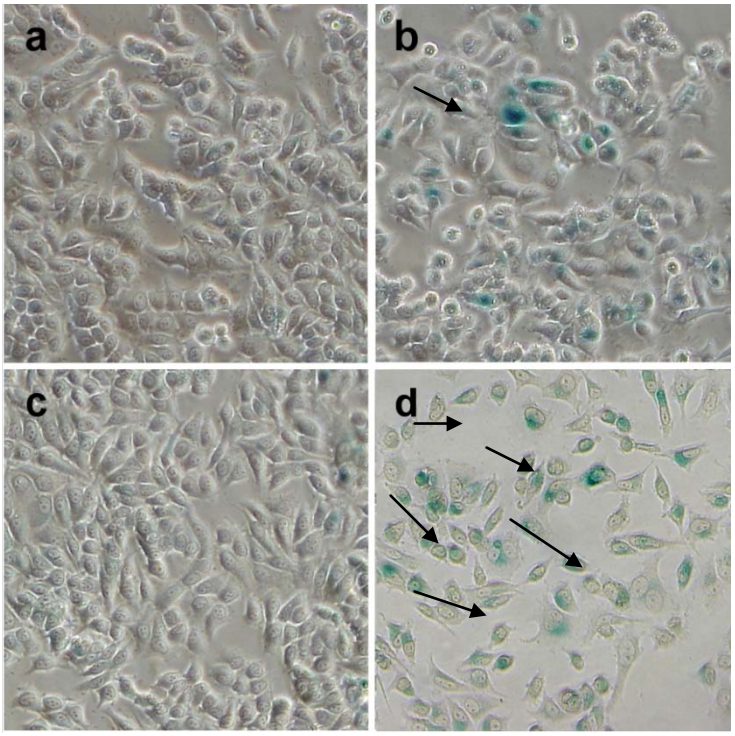


Fig 24. SA- β-gal analysis of MCF-7 cells after treatment of harmine. All cells were stained for SA- β-gal expression at day 2 and day 4 after treatment. a, untreated control MCF-7 cells; b, the SA- β-gal expression was detected at day 2; c, DMSO control; d, the SA- β-gal expression was detected at day 4. Original magnification, ×10.

4.4 HARMINE TRIGGERS P53-MEDIATED DNA DAMAGE RESPONSE IN MCF-7 CELLS

We have found that harmine treatment triggers MCF-7 cell growth arrest, at the meantime, induces cells to enter senescence status. Cellular senescence involves the action of the tumor suppressor p53. We have previously demonstrated that prolonged growth arrest and the accelerated senescence response after exposure of MCF-7 cells to a one third lethal concentration (20 μM) for 96h. Next we assessed the status of the p53 senescence pathway by a series of immunoblot analysis of crude cell lysates to figure out the underlying pathway on the harmine-induced MCF-7 cell cycle arrest. The cells were cultured with harmine in a final contration of 20 μM and then cell samples were collected at different time points (12 h, 24 h, 48 h, and 96 h). The first finding showed that the level of phosphorylation of H2AX (γH2AX) protein slightly enhanced at 48 h and jumped to near maximal levels at 96 h. Increase in p53 protein level was detected by immunoblot analysis as early as 12 h, accompanied with the increase of p21 protein, and reached near plateau levels up to 96 h (Fig 25B). c-Myc is a transcriptional enhancer of hTERT through binding onto the E-box in the hTERT promoter (Greenberg, 1999; Greenberg *et al.*, 1999; Wu *et al.*, 1999). Moreover, it has been reported that c-Myc can induce DNA damage and interfere with p53-induced cell cycle arrest (Hermeking *et al.*, 1995; Vafa *et al.*, 2002). In this study, c-Myc was determined on its protein level as well. The result of western blot showed a moderate alternation of c-Myc occurred during harmine treatment. The regulations of p53, p21, and c-Myc on protein levels were displayed in the relative intensity analysis (Fig 25C). None of the changes was observed in untreated or solvent control.

Mounted evidence showed that p21 binds to and inhibits Cdk2/cyclin E complexes thereby arresting cells proliferation (Aprelikova *et al.*, 1995; Sherr and Roberts, 1999). We then tested from transcription level whether the association of p21-CDK2 involved in MCF-7 cells that underwent harmine-induced cell cycle arrest. After harmine treatment of 20 μM, the cells were harvested at different time points (12 h, 24 h, 48 h, and 96 h). The consistent result was obtained by real-time PCR that p21 mRNA expression was up-regulated in time-dependent effect, and the up-regulation revealed as early as 12 h and reached the highest amount at 96 h. The significant inhibition of Cdk2 was detected started at 48 h and stayed stable at 96 h (Fig 25A). Taken together, all these results indicated that harmine triggers a

mild DNA damage in MCF-7 cell, and inhibits cell proliferation through the p53/p21 pathway prior to inhibiting Cdk2 activities. The result of expression of related genes was summarized in Table 10.

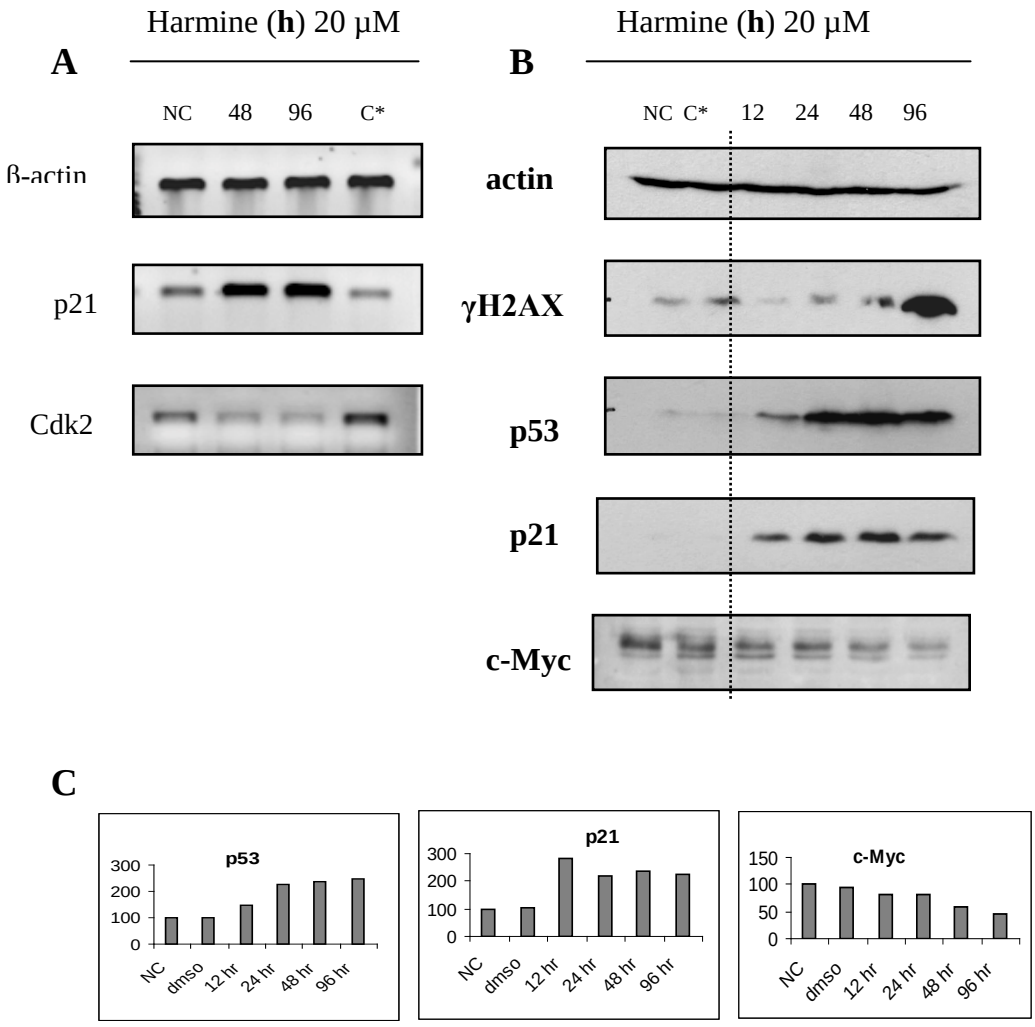


Fig 25. Increase in p53 protein level accompany mild DNA damage following prolonged exposure of harmine in MCF-7 cells. NC: untreated control; C*: DMSO control. (**A**) The total RNA extracted from MCF-7 cells that had been treated with 20 μM harmine for indicated length of time was analyzed by RT-PCR that recognize each of the indicated genes. (**B**) Total proteins extracted from MCF-7 cells that had been treated with 20 μM harmine for indicated length of time were immunoblotted with antibodies that recognize each of the indicated proteins. (**C**) Relative intensity measurement of p53, p21, and c-Myc proteins expression. The intensity values were standardized by the expression of actin and untreated control.

Table 10. The summary of relative quantification PCR result of MCF-7 cell after harmine treatment in time- and dose-effect studies.

Transcriptional regulation of genes on mRNA level						
Gene	**Time-effect (20 µM)**				**Dose-effect (48 h)**	
	12 h	24 h	48 h	96 h	10 µM	20 µM
hTERT (global)	—	—	↓	↓	—	↓
p21	↑	↑	↑	↑	—	↑
Cdk2	—	—	↓	↓		
p53	—	—	—	—		

5. DISCUSSION

5.1 The cytotoxic and anti-proliferative properties of alkaloids on human cancer cells

Alkaloids are a large group of nitrogen-containing secondary metabolites (SM). Many alkaloids possess strong toxic properties towards animals and human (Wink, 2007; 2008). The medicinal use of alkaloids could be regard as an exploitation of properties that originally had been selected and developed in an ecological or evolutionary context (Wink, 1993; Roberts and Wink, 1998). During the past decade, much attention has been drawn on the anticancer potenties of alkaloids. Alkaloids were first time introduced into clinic as early as 1965 and some of them have been used as anticancer drugs over 40 years (Johnson *et al.*, 1963; Zhou and Rahmani, 1992; Graham *et al.*, 2000).

In our study, we have evaluated the cytotoxic and anti-proliferative effect of the alkaloids harmine, emetine, and sanguinarine on human cancer cells *in vitro*. Results obtained from MTT assay showed that these compounds significant exhibited cytotoxicity against human cancer cells and induce cell cycle arrest in dose- and time- dependent manner. Cytotoxicity occurs as a result of the molecular interaction of an alkaloid with one or more important targets present in a cell (Wink, 2007), for instance, targeting DNA, RNA, or associated enzymes (Roberts and Wink, 1998; Wink, 2007;2008). This targeting will directly or indirectly lead to cell death via apoptosis (Lansiaux *et al.*, 2002; Möller *et al.*, 2007a) or senescence (Riou *et al.*, 2002; Gewirtz *et al.*, 2008).

Harmine is β-carboline alkaloid originally isolated from the plant *Peganum harmala* (Roberts and Wink, 1998; Wink and Wyk, 2008), the crude seeds of *P. harmala* are used in Chinese medicine for digestive tract tumor cure. Harmine showed a cytotoxic effect and anti-proliferative effect against human cancer cells in our research. The similar results were gained from other groups as well (Ishida *et al.*, 1999; Song *et al.*, 2004). Harmine, as reported, can repress many intracellular targets, such as cytochrome P450 (Tweedie *et al.*, 1988), and DNA topoisomerase I (Funayama *et al.*, 1996). The actual harmine-induced cytotoxicity remains elusive although it was lately reported that harmine inhibits Cdk 2 and Cdk 5 in vitro (Song *et al.*, 2002). Data from our group showed that harmine can intercalate to double stranded DNA, and whereby it interrupts DNA replication. Thus, we speculate that harmine-induced cell cycle arrest might involve such interactions *in vivo*. The cytotoxicity of

emetine was especially marked in HeLa and SiHa cells in this study. Emetine showed a stronger cytotoxic effect in cells, and the IC_{50} values are very close between cell lines. Emetine was identified as the main alkaloid of *Cephaelis acuminata* (Roberts and Wink, 1998; Wink and Wyk, 2008). In pharmacology and cell biology, emetine is frequently used as an inhibitor of protein biosynthesis in eukaryotic cells to block *de novo* protein synthesis (Grollman, 1966; Gupta and Siminovitch, 1976). The cytotoxic property of emetine has been examined since last century; it was reported to exhibit cytotoxic properties in murine tumor systems. The cytotoxicity caused by emetine has been described by caspase-dependent apoptosis in U937 (Kochi and Collier, 1993; Bicknell *et al.*, 1994), A549-S cells (Watanabe *et al.*, 2002; Möller *et al.*, 2007a), and Jurkat T-cells (Möller *et al.*, 2006). Sanguinarine is present in various plant species, but main resources are *Chelidonium majus*, *Macleaya cordata*, and *Sanguinaria canadensis* (Roberts and Wink, 1998; Dvorak and Simanek, 2007; Wink and Wyk, 2008). Sanguinarine was documented to suppress or modulate the function of various cellular enzymes, such as like NF-κB, protein kinase C (Gopalakrishna, 1995; Chaturvedi, 1997). We have observed that sanguinarine exhibited a strong cytotoxicity in cancer cells and it also significantly arrested the cell proliferation, the similar results were also found in normal cell (Malikova *et al.*, 2006a), rat hepatocytes (Choy *et al.*, 2008), as well as in many other cancer cell lines (Malikova *et al.*, 2006a). In various cell models, sanguinarine was found to cause DNA damage (Kaminskyy *et al.*, 2008; Matkar *et al.*, 2008), to modulate expression of proteins involved in regulating the cell cycle (Lee *et al.*, 2008) and apoptosis (Weerasinghe *et al.*, 2006). In some other reports, sanguinarine has been identified to reveal a strong DNA intercalating and reverse transcriptase inhibitory properties (Schmeller *et al.*, 1997), and the DNA intercalation might be responsible for the mutagenic and even carcinogenic effects of this compound (Hakim, 1968). In summary, we have shown the cytotoxicity of harmine, emetine, and sanguinarine in human cancer cells. Although all these compounds revealed strong cytotoxic effect against human cancer cells and induced cell growth arrest, the underlying mechanisms may not be the same and need to be verified in further research.

5.2 The effect of alkaloids on human telomerase

Telomerase is ribonucleoprotein complex, which is detectable in 85–90% of human cancers and over 70% of immortalized human cell lines (Kim *et al.*, 1994; Shay and Bacchetti, 1997),

and undetectable in normal somatic cells. The elevated level of telomerase has been identified as a characteristic feature of cancer cells that leads to immortality (Greider, 1998b). The evidence showed that the inhibition of telomerase activity led to the interruption of the genomic stability as well as cell growth arrest (Shay and Bacchetti, 1997). Telomerase, therefore, has been proposed as an important marker for tumorigenesis and is a potentially highly selective target for the development of anti-cancer agents (Rezler *et al.*, 2002).

The studies previously claimed that some alkaloids possess the inhibitory property on human telomerase, these alkaloids including berbamine (Ji *et al.*, 2002), chelidonine (Noureini and Wink, 2009) and 9-hydroxyellipticine (Sato *et al.*, 1998). However, little is known about harmine, emetine, or sanguinarine. In our study, we attempted to evaluate the effect of these alkaloids on human telomerase. The results obtained from TRAP assay showed that all these alkaloids displayed an inhibitory effect on human telomerase activity, but the inhibitory rates were quite different. (i) Harmine exhibited the most significant inhibition on human telomerase in MCF-7 and HeLa cell, the inhibitory rates were 82% and 87% of untreated control, respectively (Fig 11,12), whereas it caused a weaker inhibition on SiHa cell telomerase of 54% of untreated control (Fig 14). So far, the underlying mechanisms related to this result hve not been documented yet. However, results obtained from different groups showed that harmine is a promising cell proliferation inhibitor against cancer cells and significantly inhibits the activity of Cdk2 and Cdk4 in vitro (Song *et al.*, 2004; Jimenez *et al.*, 2008). During the past years, many studies have tried to build up a linkage between telomerase expression and the sensitivity of cytotoxic drugs, but no direct correlation has been discovered till now (Akiyama *et al.*, 1999; Cressey *et al.*, 2002). Our observation is suggesting the cellular cytotoxicity which is caused by harmine is due, at least partially, to the inhibition of telomerase in those cancer cells. (ii) Emetine revealed a moderate inhibition on HeLa cell telomerase, and had a weak inhibitory effect on SiHa cells. Emetine, as reported possess strong toxicity, the data obtained from our other research showed that emetine is a promising apoptosis inducer in leukemia cells (Möller *et al.*, 2007a; Möller and Wink, 2007b). Some researchers have argued that telomerase might play a role in cellular resistance to apoptosis. For example, cisplatin known as telomerase inhibitor (Burger *et al.*, 1997) triggered a halt of cell-cycle progression, telomere loss, and apoptosis (Ishibashi and Lippard, 1998). Even in some case, without altering the telomerase activity, the cells undergoing

apoptosis after treatment of paclitaxel in murine melanoma cells (Multani *et al.*, 1999) and in human pharynx cancer cells (Mo *et al.*, 2003). Therefore, we speculate that the signaling pathway of cellular apoptosis may explain the inhibition of cell proliferation (Fig 10c,d) and telomerase activity which were induced by the emetine treatment. (iii) The alkaloid sanguinarine is known for its anticancer properties and possesses the potential for selective elimination of cancer cells.

Telomeres are located on the each end of chromosome, protecting chromosome ends from fusion, recombination problems. It has recently been suggested that within a population of cells there are large variations in telomere lengths, and the shortest telomeres are the most sensitive and lethal for cell viability (Hemann *et al.*, 2001). Single stranded telomeric DNA sequences can be driven by the formation of guanine-quartets, forming an inter- or intramolecular G-quadruplex (Williamson, 1994; Simonsson, 2001). The human single-stranded telomere end, which can potentially fold into a number of four-repeat quadruplexes, is far longer and more stable than in many other vertebrates, and mainly form two types of intramolecular G-quadruplex in vitro (Parkinson *et al.*, 2002). A dynamic equilibrium at telomere ends between single-stranded and various types of folded telomeric repeats has been demonstrated (Griffith *et al.*, 1999), telomerase itself will force this equilibrium towards single-strandedness at replication, since only then can template hybridisation and the synthesis of telomeric repeats on to 3' ends take place (Cuesta *et al.*, 2003). A number G-quadruplex ligands have been reported are able to inhibit telomerase through quadruplexes binding, termed intercalation (Cuesta *et al.*, 2003). The potential role of quadruplexes *in vivo* has been highlighted with the recent development of therapeutic strategies designed to stabilize telomeric ends as G-quadruplex structures using specific small molecules, which can destabilize telomere maintenance in tumour cells (Parkinson *et al.*, 2002).

Alkaloid-based ligands like berberine and its synthetic derivative have been examined for G-quadruplex binding and their ability to inhibit telomerase (Franceschin *et al.*, 2006). Results show that these molecules have selectivity for G-quadruplex compared with duplex DNA, and that their aromatic moieties play a dominant role in quadruplex binding (Amit *et al.*, 2010). The selected alkaloids harmine, emetine, and sanguinarine have been investigated in our lab previously. The results obtained from our group and other groups showed that they are ability to intercalate DNA *in vitro* (Duportail, 1981; Schmeller *et al.*, 1997; Wink *et al.*,

1998; Wink, 2007), the strongest effects were detected for sanguinarine, followed by harmine, and emetine (Wink *et al.*, 1998). However, in this study, we have found that harmine displayed a stronger inhibition on HeLa telomerase than that of sanguinarine, and the repression appeared obviously as early as 24 h (Fig. 12 and 13). One may argue that the inconsistent results might base on the low concentration of sanguinarine assessed in telomerase assay. The selected concentrations of harmine and sanguinarine for TRAP assay were according to the cell survival assay, in order to ensure the cell viability remaining 70% under treatments of both alkaloids (Table 9), and also different types of cell showed various sensitivities in response to the treatment of same compound. Data obtained from the half-life assessment indicates that telomerase is a highly stable molecule, with a half life > 24 hours in general (Holt *et al.*, 1997). Shawn. *et al* have made a conclusion after evaluating the relationship between cell cycle regulation and telomerase activity in different human cancer cells, that telomerase activity generally correlates with growth rate and is repressed in cells that exit the cell cycle (Holt *et al.*, 1997). Therefore, we speculate that harmine may trigger additional mechanisms simultaneously to facilitate the inhibition of telomerase activity in HeLa cell. The regulation and/or repression may thus include mechanisms such as the direct physical interaction of telomerase with regulatory proteins or degradation of the RNA or protein components. Moreover, all these compounds showed an inhibitory effect on reverse transcriptase *in vitro* within a range from 0.03 mM to 0.5 mM (Wink *et al.*, 1998). Taken together, all our data suggest that the alkaloids harmine, sanguinarine, and emetine can partially inhibit human telomerase through a fashion of intercalation, although the direct evidence of *in vivo* study needs to be further proved.

5.3 The transcriptional regulation of hTERT expression in MCF-7 and HeLa cells

Among all subunits of telomerase, the most important component responsible for the enzymatic activity of telomerase is hTERT (Meyerson *et al.*, 1997; Nakamura *et al.*, 1997). A tight link has been established between the transcriptional level of hTERT and telomerase activity (Bodnar *et al.*, 1998; Takakura *et al.*, 1998; Kyo *et al.*, 1999). Therefore, to evaluate the regulatory expression of hTERT will lead to a better understanding of telomerase regulation in human tumors. Due to the fact that harmine induced a severe decrease of telomerase activity in both MCF-7 and HeLa cells, therefore, we preceded our research in the further step on these two cell lines.

We have detected previously that harmine exposure led to a dramatic suppression of telomerase activity in MCF-7 and HeLa cells, respectively. In MCF-7 cells, the significant inhibition of telomerase activity was observed at 96 h, it was at least 24 h after down-regulation of hTERT mRNA expression, which was detected at 48 h. This is consistent with the observation that the half-life of hTERT is approximately 24 h (Holt *et al.*, 1996b) and suggests that telomerase repression is achieved at the transcriptional level of hTERT. However, no alternation of hTERT on mRNA level was observed in HeLa cells.

Transcriptional activation has been considered a major mechanism for the regulation of the hTERT gene (Poole *et al.*, 2001). However, the data obtained from our research and previous observations (Kim *et al.*, 1994; Shay and Bacchetti, 1997) showed that the expression of hTERT does not explain every event of the inhibition of telomerase activity caused differently. Also, the presence of hTERT mRNA encoding functional or defective spliced isoforms has been demonstrated in several types of human cells (Kilian *et al.*, 1997; Ulaner, 1998; Ulaner *et al.*, 1998; Liu *et al.*, 1999). The splicing pattern of hTERT mRNA has been considered as part of a regulatory mechanism for telomerase activity, but the details are unclear. Only three spliced isoforms could be detected in our study, the full length, the α deletion, and the β deletion isoforms, the -α-β variant was permanently absent throughout this research. We found that the expression of all detectable isoforms of hTERT was decreased in MCF-7 cell (Fig 22), whereas in HeLa cells, the β deletion isoform was increased, the full length was slight decreased compared with untreated or solvent control, the expression of α deletion isoform was not altered (Fig 23). The β deletion variant has been observed in various cell types (Nakamura *et al.*, 1997; Wick *et al.*, 1999). It lacks two exons in its coding region, leading to a frameshift and premature termination of translation, resulting in dysfunction of the protein (Kilian *et al.*, 1997). Taken together, our research has found that harmine exert its inhibitory effect on human telomerase activity through different mechanisms depending on the cell type.

5.4 The regulation of the expression of hTERT mRNA

The results obtained in our study showed that p53 protein was overexpressed during harmine exposure, the increase displayed as early as 12 h and stayed stable throughout the entire treatment period. Such alternation accompanied with an overexpression of p21 and an

eventual decrease of c-Myc as well (Fig 25). At the meantime, the result of semi-quantification PCR showed that the significant repression of the hTERT expression was detected at 48 h, it was at least 24 h later than the overexpression of p53, which suggest that MCF-7 cells lacking hTERT expression were more sensitive to cell cycle arrest and/or cell death induced by harmine. Moreover, the overexpression of wild-type p53 was observed to down-regulate telomerase enzymatic activity through transcriptional repression of hTERT independent of its effects on cell growth arrest and apoptosis (Gollahon *et al.*, 1998; Kusumoto *et al.*, 1999). Moreover, normal human breast epithelial cells transfected with a p53 mutant became immortalized and were reactivated for telomerase (Gollahon and Shay, 1996), which suggest the existence of a p53-dependent regulatory pathway for hTERT/telomerase control in human cells. Thus, we argued that the hTERT expression is transcriptionally downregulated in our study, at least partially, upon the induction of wt p53 in MCF-7 cells. Similarly, two recent studies showed that the overexpression of wt p53 inhibited telomerase activity and hTERT expression in pancreatic cancer cells *(*Kusumoto *et al.*, 1999; Kanaya *et al.*, 2000).

p53 can interact with different transcription factors or protein components to direct its transcriptional repression, such as the TATA binding protein (TBP) (Martin *et al.*, 1993; Horikoshi *et al.*, 1995), CCAAT-binding factor (CBF) (Matuoka and Chen, 2002) and Sp1 (Gualberto and Baldwin, 1995; Bargonetti *et al.*, 1997). Sp1 acts as a media in the interaction between p53 and hTERT. hTERT promoter does not contain any p53 binding sites due to the lacking of TATA motif. However, the serial deletion assays revealed that within the 181-bp core region of hTERT promoter that contains five GC-boxes (Takakura *et al.*, 1999), Sp1 can transactive hTERT by directly binding onto these GC-boxes (Kyo *et al.*, 2000). Abrogation of Sp1 bindings by substitution mutations resulted in a marked loss of transcriptional activity indicating that Sp1 is a critical transactivator possibly involved in the basal transcription of hTERT (Kanaya *et al.*, 2000). The overexpressed p53 promote the formation of p53-Sp1 complexes, which disrupt Sp1-hTERT promoter binding and unable Sp1 to transactivate the hTERT (Xu *et al.*, 2000). The direct evidence of formation of p53-Sp1 complex was obtained by co-immunoprecipitat technique (Xu *et al.*, 2000).

Except Sp1 binding sites, two canonical E-box elements were identified within the core region of hTERT promoter (Cong *et al.,* 1999; Horikawa *et al.,* 1999). Several groups have reported that c-Myc protein was able to bind the E-box directly by the electrophoretic mobility shift assay (EMSA) (Wu *et al.,* 1999; Kyo *et al.,* 2000). In this study, we have found that the down-regulation of hTERT expression accompanied by a gradual decrease of c-Myc protein in MCF-7 cells (Fig 25), and the similar phenomenon was appeared in the research as well (Cerezo *et al.*, 2002). Different studies have found that overexpression of c-Myc protein led to a remarkable, E-box dependent increase in the hTERT promoter activity (Wu *et al.,* 1999; Kyo *et al.,* 2000). Moreover, the overexpressed c-Myc could induce the expression of endogenous hTERT mRNA and telomerase activity in normal human cells (Wang *et al.* 1998; Greenberg *et al.,* 1999), indicates c-Myc plays an important role in the transcriptional regulation of hTERT.

We observed a down-regulation of hTERT transcription in MCF-7 cells after harmine treatment and that did not occur in HeLa cells, instead, harmine induced a frameshifting by the post-transcriptional regulation leading to a slight up-regulation of β variant of hTERT. So far, the underlying mechanisms of alternative splicing are not yet clear. Recent study showed that the oncoprotein E6 was able to induce the transcriptional activation of hTERT (Klingelhutz *et al.,* 1996). E6 is known to bind p53 and to promote its degradation in cervical carcinoma cells (Scheffner *et al.,* 1990; Klingelhutz, 1996). Some of the cellular E6-interact proteins, such as E6TP1 and E6AP (Gewin and Galloway, 2001) are speculated to be involved in the hTERT activation as well (Gao *et al.,* 2001). Therefore, we speculated that E6 and its associated factors might be involved in telomerase regulation in HeLa cells in response to harmine treatment, but together with the molecular mechanism of the alternative splicing of hTERT need to be further investigated.

5.5 Harmine induces a p53-associated DNA damage response in MCF-7 cells

The result of western blot showed that the expression of γH2AX was significantly increased at 96 h in MCF-7 cells (Fig 25). The increase in γH2AX expression is an early sign of genomic event reflects induction of double strands breaks (Albino *et al.,* 2004; Tanaka *et al.,* 2007). This finding suggests that harmine triggered DNA damage response in MCF-7 cells, and this hypothesis is confirmed by other investigation although harmine was used at doses

greater than in the present study. Boeira and Moura in their reports claimed that harmine was able to induce significant strand breaks in V79 cell by using the chromosome aberration test (Boeira *et al.*, 2001; Moura *et al.*, 2007). Moreover, harmine was also characterized to enhance the induction of chromosome aberrations and produce DNA breakage in mammalian cells in cultured mammalian cells (Sasaki *et al.*, 1992; Boeira *et al.*, 2001) by the intercalation of DNA (Meester, 1995; Taira *et al.*, 1997; Balon, 1999). The same pattern of interaction was observed in our previous study as well (Wink, 2007). Moreover, harmine has been also identified to able to inhibit topoisomerase I (Cao *et al.*, 2005; Wink, 2007) and blocking the enzyme from the breakage-rejoining action to repair DNA damage and fix mutations (Sasaki *et al.*, 1992; Wang, 1998), though which to favor DNA strand breaks.

Different studies implicated that p53 is a critical determinant of cell fate following certain types of DNA damage (Clarke *et al.*, 1993; Liu and Kulesz-Martin, 2001). The p53 protein can sense DNA strand breaks and to halt cell cycle progression at G_1 check point by activating p21 transcription on DNA damage signal (el-Deiry, 1993). In our study, a rapid increase of p53 was observed in MC-7 cells following the treatment of harmine. The overexpression was revealed as early as 12 h and stayed stable throughout the entire treating period (96 h). Such elevation accompanied by an induction of p21 on both mRNA level and protein level (Fig 25), the inhibitor of cycling-dependent kinase (Cdk). Based on the previous demonstration that the p53-induced increase of p21 during DNA damage only inhibits Cdk2 activity, the inhibition of Cdk4 is through a different mechanism (Boulaire *et al.*, 2000; Bartek and Lukas, 2001; Agami and Bernards, 2002). Therefore, we only investigated the expression of Cdk2 in harmine treated MCF-7 cells. The result showed that the expression of Cdk2 mRNA was repressed just as what we expected. This result is consistent with the previous studies that DNA damage-induced Cdk inhibition can be caused by induction of the Cdk inhibitor p21 by p53 (Eldeiry *et al.*, 1993; Dulic *et al.*, 1994). However, the decrease expression of Cdk2 mRNA could also be initiated through a direct inhibitory effect by harmine, because it has been claimed by Song et al. that harmine is specific inhibitor of Cdk2 in a low dose by enzymatic assay *in vitro* (Song *et al.*, 2004). Taken together, we conclude that harmine under our tested dose triggers DNA damage in MCF-7 cells and initiate cell cycle arrest via p53/p21 pathway.

5.6 Harmine initiates a DNA damage response and induces MCF-7 cells enter premature senescence

Accelerated senescence is characterized by the rapid induction of an irreversible growth arrest and some morphological features, such as enlarged nuclear and flattened cell shape (See chapter 1.3). We have previously showed the permanent growth arrest of MCF-7 cells during the chronic treatment of harmine for 4 days, resulted in accelerated senescence based on β-galactosidase expression and cell morphology (Fig 24).

A number of studies have described a state of accelerated senescence or stress-induced premature senescence in response to various treatments that induce DNA damage including ionizing radiation (Oh *et al.*, 2001; Gorbunova *et al.*, 2002), ultraviolet (UV) radiation (Chainiaux *et al.*, 2002; Gorbunova *et al.*, 2002), hydrogen peroxide generation *(*Gorbunova *et al.* 2002; Frippiat *et al.*, 2003) and antitumour drugs such as cisplatin, adriamycin and camptothecin (Chang *et al.*, 1999a; Elmore *et al.*, 2002; Wang *et al.*, 2004). It has been demonstrated that the signaling pathways driving replicative senescence and DNA damage-induced accelerated senescence are very similar (Gewirtz *et al.*, 2008). Both events involve the action of tumor suppressor p53 and pRB. Furthermore, some studies claimed the induction of accelerated senescence response was p53-dependent (Suzuki *et al.*, 2001; Elmore *et al.*, 2002). Harmine can induce apoptosis in a variety of experimental human cancer cell lines (Chen *et al.*, 2005; Song *et al.*, 2006; Jimenez *et al.*, 2008). However, in this study, the result of flow cytometric analysis (data not shown) demonstrated that MCF-7 cells failed to undergo a primary apoptotic response; instead cells entered an accelerated senescence after the chronic exposure to harmine. Considering MCF-7 cells express wild-type p53 but lack functional caspase 3 protein (Kurokawa *et al.*, 1999), the induction of senescence during harmine treatment might be attributed to a combination of p53-mediated senescence and the inability to progress down the apoptotic pathway. Similar results obtained from other studies (Jones *et al.*, 2005; Elmore *et al.* 2002), MCF-7 cells failed to undergo apoptotic cell death but underwent accelerated senescence after the exposure of ionizing radiation and adriamycin. However, when p53 protein was diminished by infection of HPV-E6 oncogene , MCF-7-E6 cells entered delayed programmed cell death (Elmore *et al.*, 2002). Different to replicative senescence, telomere shortening was rarely detected in accelerated senescence; instead, it was more associated with telomere dysfunction and/or alterations of

telomerase activity (Leteurtre *et al.*, 1997; Joo *et al.*, 1998; Neuhof *et al.*, 2001; Wang *et al.*, 2004). In our study, although the telomere length of MCF-7 cells was not examined after harmine exposure, considering the terminal restriction fragment (TRF) assay only measures average telomere lengths and not individual telomeres, it is formally possible that a single shortened telomere could be responsible for the senescence response (Hemann *et al.*, 2001; Von Zglinicki, 2003). Therefore, we speculate that the senescence induced by harmine was likely to attribute to the reduction in the protective function of the chromosome ends. As a result of harmine treatment, the chromosomal ends (especially the G-quadruplex structures) were preferentially targeted for DNA damage. Moreover, harmine as reported is able to inhibit the activity of topoisomerase I (Song *et al.*, 2004; Wink, 2007), where single strand breaks accumulate and/or the stabilization of G-quadruplexes force deprotection at the telomere.

6. OUTLOOK

Alkaloids comprise a class of secondary metabolites (SM) with fascinating properties. They are not waste products but have evolved mainly as defense compounds against herbivores, also against microbes, competing other plants, and even against viruses. In this study, the alkaloids harmine, emetine, and sanguinarine have shown to elicit their anticancer properties via a number of mechanisms.

- Cellular cytotoxicity
- Anti-proliferative activity
- Inhibit human telomerase
- Modulating the gene expression through different pathways
- Inducing cells enter premature senescence status

However, much more remains unknown. For focusing on specific targets, there are still some works can be done based on these finding. For example, to analysis MCF-7 cell cycle was stopped at which phase? Although we have gained some results by using FACS assay, they still need to be further proved. Also, what are the potential mechanisms which trigger an alternative splicing shift of hTERT expression in HeLa cell?

In general, there is an increasing interest in the discovery of novel antitumor agents from natural resources. Over 46% of newly approved drugs and new drug candidates for cancer therapy by Food and Drug Administration were of natural origin during the period from 1989 to 1995. The medicinal use of alkaloids and other natural products could be concerned as exploitation of properties that originally had been selected and developed in an ecological or evolutionary context (Wink, 1993; Roberts and Wink, 1998). The right plants and concentrations become excellent means for the therapeutic treatment of illness, disease, and even cancer. Many cytotoxic compounds, such as pyrroles, pyrazines, imidazoles have been tested as novel antitumor agents (Wink, 2007).

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